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Quantitative proteome analysis by solid-phase isotope tagging and mass spectrometry

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The adaptation of sequences of chemical reactions to a solid-phase format has been essential to the automation, reproducibility, and efficiency of a number of biotechnological processes including peptide and oligonucleotide synthesis and sequencing[1–4]. Here we describe a method for the site-specific, stable isotopic labeling of cysteinyl peptides in complex peptide mixtures through a solid-phase capture and release process, and the concomitant isolation of the labeled peptides. The recovered peptides were analyzed by microcapillary liquid chromatography and tandem mass spectrometry (μLC-MS/MS) to determine their sequences and relative quantities. The method was used to detect galactose-induced changes in protein abundance in the yeast *Saccharomyces cerevisiae*. A side-by-side comparison with the isotope-coded affinity tag (ICAT) method[5] demonstrated that the solid-phase method for stable isotope tagging of peptides is comparatively simpler, more efficient, and more sensitive.

We devised a method for site-specific, stable isotopic labeling of cysteinyl peptides using a solid-phase isotope tagging reagent (Fig. 1). The *o*-nitrobenzyl-based photocleavable linker was first attached to aminopropyl-coated glass beads by solid-phase peptide synthesis[6]. Next, the isotope tag, a leucine molecule containing either seven hydrogen (d0) or seven deuterium atoms (d7), was attached to the

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photocleavable linker, again by solid-phase peptide synthesis[6]. Finally, a sulfhydryl-specific iodoacetyl group was attached. Cysteinyl peptides from two samples to be compared were covalently captured on the solid phase containing isotopically heavy or normal tag. The beads were then combined, washed, and exposed to UV light (360 nm, chosen to minimize any possible photocatalyzed side reactions). This resulted in photocleavage of the linker and the transfer of isotope tags from the solid phase onto the side chain of cysteine residues. Finally, recovered tagged peptides were analyzed by μLC-MS/MS to determine the sequence and relative abundance of each peptide, essentially as described previously[5].

To illustrate the efficiency of the capture and release reactions, we used a mixture consisting of a cysteine-containing laminin B peptide and the non-cysteine-containing phosphoangiotensin (Fig. 2). Laminin B was quantitatively captured onto the solid phase (compare Fig. 2A, 2B). After 1 h of photocleavage, the tagged laminin B was recovered; it showed the expected mass
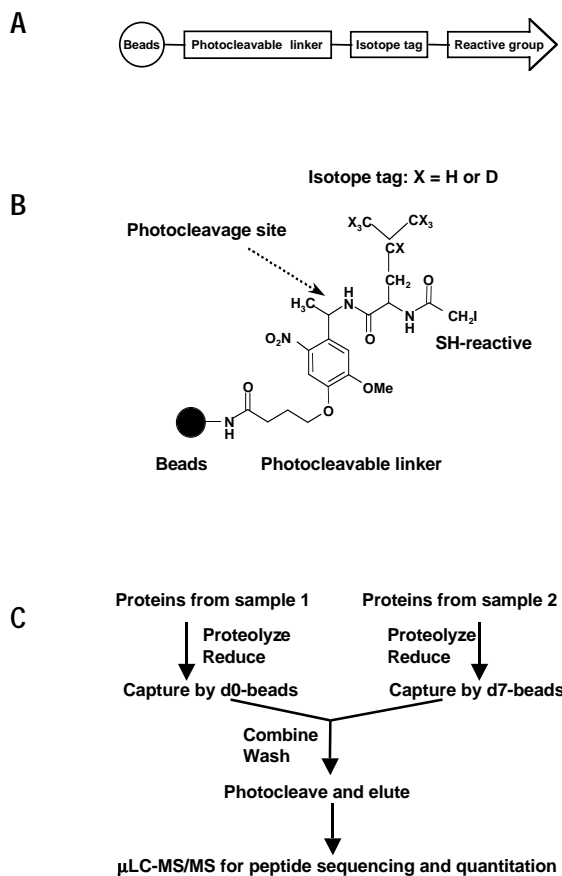

Figure 1. Schematic representation of the solid-phase isotope tagging method. (A) Modular composition of the solid-phase isotope tagging reagent, consisting of four elements: beads, photocleavable linker, stable isotope tag, and specific reactive group. (B) Chemical composition of the sulfhydryl (SH)–reactive solid-phase isotope tagging reagent. The *o*-nitrobenzyl-based photocleavable linker was coupled to aminopropyl glass beads. Peripheral to the photocleavable linker, a leucine molecule containing either 7 hydrogen (H) or 7 deuterium atoms (D), indicated by 'X', was attached as the isotope tag, followed by an iodoacetyl group as the SH-reactive group. (C) Strategy for quantitative protein analysis. Two protein samples to be compared were subjected to proteolysis. The Cys-containing peptides were reduced and captured by beads carrying either the d0-leucine or d7-leucine tag. The beads were then combined and, after stringent washing of the beads, the tagged peptides were released by photocleavage and analyzed by μLC-MS/MS.

modification (+170 Da) due to the addition of leucine tag to the cysteine residue (Fig. 2C), which was also confirmed by MS/MS (data not shown). The hydrophobic nature of the leucine tag increases the retention time of tagged laminin B as compared with the untagged form. The signal intensities of untagged and tagged laminin B, contrasting with the identical amounts of phosphoangiotensin, indicated specific capture and almost complete recovery of tagged laminin B. Longer photocleavage time did not affect either the yield or the quality of the tagged laminin B (data not shown), indicating that photocatalyzed side reactions did not occur to any substantial extent.

Stable isotope tagging is a general strategy for quantitative analysis of proteins by mass spectrometry[5,7], exemplified by the previously published ICAT method[5,8,9]. To compare the performance of the solid-phase approach with that of the standard ICAT approach, we performed a side-by-side comparison in which the two methods were used to detect protein expression changes in the yeast *S. cerevisiae* in response to induction with galactose. Two different amounts of starting protein material were evaluated (100 μg large sample load and 10 μg small sample load of the same yeast proteins). Because our objective was to compare the relative performance of the labeling strategies, and not the peptide separation or protein identification strategies, we performed single LC-MS/MS runs on all samples for protein identification. This minimized variations in the results due to sample processing rather than labeling, but probably resulted in the identification of fewer proteins than would be obtainable with larger sample sizes and optimized peptide separations upstream of LC-MS/MS[9,10].

In both the small- and large-scale experiments, the number of proteins identified and quantified by the solid-phase method was greater than by ICAT (Fig. 3; for full lists of the proteins quantified, see Supplementary Tables 1–4 online). Indeed, the solid-phase approach was more sensitive, identifying the majority of the proteins identified by conventional ICAT in addition to many others not identified by ICAT (Fig. 3A, 3B). Quantification of the same proteins identified in multiple experiments was also consistent (see Supplementary Tables 1–4 online). Thus, protein quantification was not influenced by either the structure of the isotope tag or the capture and release chemistry of the solid-phase method.

Galactose is known to strongly induce expression of several genes involved in galactose utilization, including galactokinase (GAL1), galactose permease (GAL2), galactotransferase (GAL7), and UDP-glucose-4-epimerase (GALX)[11]. After induction with galactose, we identified and quantified multiple peptides from proteins including GAL1, GAL2, GAL7, and GALX by the solid-phase method (Supplementary Tables 1–4 online). In contrast, using the ICAT method we identified only one peptide from the GAL1 protein in both the small- and large-sample-load experiments, and found a lower signal-to-noise ratio than with the solid-phase method (data not shown). These data confirmed the superior sample recovery and sensitivity of the solid-phase over the ICAT approach. In addition, the reproducibility of the solid-phase method was demonstrated by the substantial overlap in proteins identified by the small- and large-scale experiments (Fig. 3C).

The data presented here show that the solid-phase method is simple, reproducible, efficient, and sensitive for quantitative protein analysis. Indeed, as compared with the ICAT method, it has several advantages. First, both the isolation of cysteine-containing peptides and the stable incorporation of isotopes are achieved essentially in a single step. Thus, the solid-phase method is faster and simpler, requiring less manual input than the ICAT approach. Second, the covalent capture of peptides to a solid phase permits the use of stringent wash conditions to remove non-covalently associated molecules. Indeed, the experiments presented here resulted in the recovery of almost exclusively cysteinyl peptides. Third, this procedure is unaffected by the presence of proteolytic enzymes such as trypsin or of strong denaturants and detergents
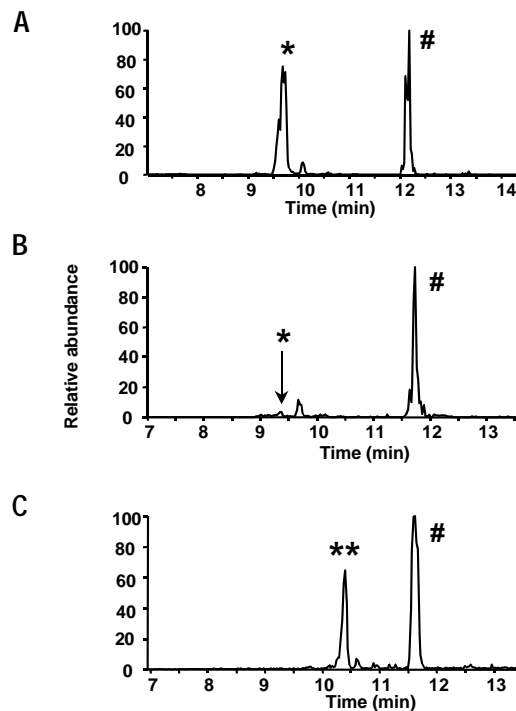

Figure 2. Validation of the solid-phase capture and release method. LC-MS analysis of a peptide mixture consisting of a cysteinyl peptide laminin B (*), the non-cysteine-containing phosphoangiotensin (#), and the isotopically tagged laminin B peptide (**) at various stages of the capture and release process. Solid-phase isotope tagging reagent with d0-leucine as isotope tag was used. (A) Ion chromatogram of laminin B (*, m/z = 484, 2+ ion) and phosphoangiotensin (#, m/z = 563.5, 2+ ion) before binding to the beads. (B) Ion chromatogram of the supernatant after 5-min binding reaction, indicating a virtually complete disappearance of laminin B signal relative to the persisting signal for phosphoangiotensin. (C) Photocleaved product generated after 1 h of illumination. The tagged laminin B (**, m/z = 569, 2+ ion) was quantitatively recovered. Identical amounts of phosphoangiotensin were present in the sample at each stage for comparison.

such as urea and SDS. There is therefore no need for additional steps to remove such molecules. Because of the minimal sample handling, this solid-phase method is more sensitive than the ICAT method. As many biologically interesting events involve relatively low-abundance regulatory proteins, the solid-phase method should be useful for the analysis of induced changes to such proteins. Fourth, the standard solid-phase peptide chemistry involved in the coupling process allows the use of a range of natural or unnatural amino acids as the isotopic mass tag in place of the d0/d7-leucine used here. This could facilitate the synthesis of beads with a range of mass tags for analysis of multiple samples (more than two) in a single experiment. Fifth, the mass tag on the cysteine used here weighs 170 Da for the d0-leucine tag. Because of the small size and the chemical nature of the tag, the observed peptide fragmentation in the MS/MS mode was not complicated by undesirable fragmentation of the label itself (data not shown), in contrast to the situation with ICAT-labeled peptides[9]. Finally, before photocleavage, the covalently immobilized peptides provide ideal substrates for additional chemical and enzymatic reactions if desired[12].

One significant way that the solid-phase method differs from the ICAT method is that the solid-phase reagent labels peptides after proteolysis, whereas in ICAT, proteins are labeled before proteolysis. Therefore, the ICAT approach is preferred in cases where separation of labeled proteins is required, such as gel electrophoresis. The solid-phase method presented here should, however, provide a tool suitable for general application to quantitative proteomics and amenable
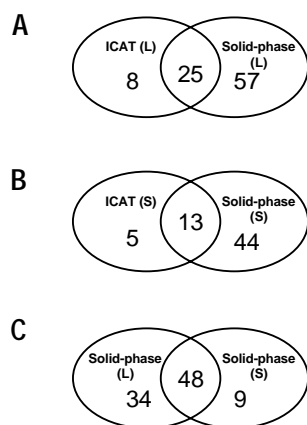
A

ICAT (L) 8 | 25 | Solid-phase (L) 57

B

ICAT (S) 5 | 13 | Solid-phase (S) 44

C

Solid-phase (L) 34 | 48 | Solid-phase (S) 9

Figure 3. Summary of the number of proteins identified and quantified by the solid-phase and ICAT methods. (A) Number of proteins identified from large-scale experiment (L), in which 100 µg total protein sample was labeled and 20 µg was analyzed by µLC-MS/MS. We quantified 82 proteins by the solid-phase method and 33 proteins by the ICAT method, with 25 proteins in common. (B) Number of proteins identified from small-scale experiment (S), in which 10 µg of total sample was labeled and 5 µg analyzed. We quantified 57 proteins by the solid-phase method and 18 proteins by the ICAT method, with 13 proteins in common. (C) Number of proteins identified by the solid-phase method in large-scale (L) and small-scale (S) experiment. Numbers in segments of Venn diagrams indicate the numbers of proteins quantified.

to automated implementation. It thus represents a step forward towards much wider applications of stable isotope tagging for quantitative protein analysis by mass spectrometry.

Experimental protocol

Synthesis of the solid-phase isotope labeling beads. Unless otherwise noted, chemicals were purchased from Aldrich (Milwaukee, WI). First, 0.5 g of aminopropyl-coated controlled-pore glass beads (amine content ~400 µmol/g; Sigma, St. Louis, MO) were washed with anhydrous dimethylformamide (DMF). Then 600 µmol each of 1-hydroxybenzotriazole (HOBt; Nova Biochem, Laufelfingen, Switzerland), Fmoc–aminoethyl photolinker (Nova Biochem), and diisopropylcarbodiimide (DIC) were mixed for 30 min at room temperature, and this mixture was added to the beads for 90 min. The beads were then washed sequentially with DMF and dichloromethane and capped with 2 ml 40% acetic anhydride/60% pyridine in dichloromethane for 30 min. The beads were washed again with DMF and treated with 20% piperidine in DMF for 30 min to remove Fmoc protection. This process was repeated to attach Fmoc–leucine (Nova Biochem) as the isotope tag. Finally, the iodoacetyl group was attached to the beads as described elsewhere[12]. Beads were washed successively with DMF, water, and methanol, dried under reduced pressure, and stored at room temperature in the dark. For synthesis of beads with heavy isotope, Fmoc–d7-leucine was prepared from d7-leucine (Isotec, Miamisberg, OH) and Fmoc–N-hydroxysuccinimide (Nova Biochem) according to a published protocol[13] except that the recrystallization step to purify Fmoc-leucine was omitted.

Yield determination of solid-phase capture and photo cleavage reactions. A sample consisting of 1 nmol of cysteine-containing laminin B peptide (sequence, CDPGYIGSR) and 500 pmol non-cysteine-containing phosphoangiotensin (sequence, DRVY*IHPF, with asterisk indicating phosphorylated tyrosine) was used. Peptides were reduced with 5 mM tris(carboxyethyl) phosphine (TCEP) in 100 µl of 0.2 M Tris (pH 8.0), 10 mM EDTA for 30 min at room temperature. Five milligrams of the beads prepared as described above (2 µmol binding capacity) were used to capture peptides in a volume of 100 µl under constant agitation. Aliquots (1 µl) of the supernatant were removed from the reaction mixture for µLC-MS analysis before addition of the beads and at different time points after their addition. After 15 min of incubation, the beads were inactivated by the addition of 2 µl of β-mercaptoethanol for 5 min and washed sequentially with 2.0 M sodium chloride,

methanol, and water. For photocleavage, the beads were resuspended in 100 µl of 0.2 M Tris (pH 8.0), 10 mM EDTA, 2% β-mercaptoethanol. Phosphoangiotensin (500 pmol) was added as an internal standard. Light from the Blak-Ray long-wave UV lamp (100 W, VWR Scientific, West Chester, PA) was filtered by 10% copper (II) sulfate solution (1 cm path length) and used to illuminate the beads from a distance of 10 cm. At different time points of illumination, an aliquot of 1 µl was taken from the supernatant for µLC-MS analysis as described below. Beads were occasionally agitated to ensure uniform light illumination. The use of β-mercaptoethanol in the photocleavage buffer prevents possible methionine oxidation during photocleavage.

Preparation of yeast protein samples. Yeast strain BY4742 was grown in either 100 ml YPR (1% yeast extract, 2% peptone (Difco, Detroit, MI), and 2% raffinose) or YPR + 2% galactose to an A_{600} of 1. Spheroplasts were prepared as described[14]. Spheroplasts were lysed in 50 mM Tris (pH 8.0), 5 mM EDTA, 6 M urea, 0.5% SDS. Cell lysate was centrifuged at 15,000g (14,000 rpm) for 15 min. The supernatant was collected and desalted on an Econo-Pac 10DG column (Bio-Rad, Hercules, CA) in 50 mM Tris (pH 8.0), 5 mM EDTA. Protein concentration was determined by Bio-Rad protein assay.

Isolation and isotope labeling of tryptic digest of yeast proteins by the solid-phase capture–release method. For the larger-scale experiment, 50 µg of each protein extract (100 µg combined) from yeast cells grown with or without galactose was prepared in 100 µl of 200 mM Tris (pH 8.0), 5 mM EDTA. Each protein extract was digested by 5 µg trypsin for 3 h at 37°C and reduced with 5 mM TCEP, and cysteinyl peptides were then captured by beads with either d0- or d7-leucine tag for 15 min as described above. The beads were combined and washed, and labeled peptides were released by 2 h of light illumination. The released peptides were loaded on an MCX column (Waters, Milford, MA) and washed sequentially with 4 ml 0.1% trifluoroacetic acid (TFA), 4 ml 80% acetonitrile/0.1% TFA, and water (to neutralize). Peptides were eluted by 1 ml of a mixture of 9 volumes methanol and 1 volume 28% ammonia, and dried under reduced pressure. Dried peptides were resuspended in water for µLC-MS/MS analysis. Next, 20% of the recovered peptides (representing 20 µg of combined proteins) were analyzed by µLC-MS/MS using a LCQ ion-trap mass spectrometer (Finnigan, San Jose, CA) as described[12]. Protein identification and quantification were performed with Sequest and existing software[9,15]. For the small-scale experiment, 5 µg of each protein extract from cells grown with or without galactose was digested by 0.5 µg trypsin and processed as described above, and then 50% of the peptide mixture recovered (representing 5 µg of combined protein extracts) was analyzed by the same µLC-MS/MS method.

Isotope labeling of yeast proteins and isolation of peptides by ICAT. As starting material, 100 µg of each protein extract from cells grown with or without galactose was prepared in labeling buffer containing 200 mM Tris (pH 8), 0.5 mM EDTA, 6 M urea, and 0.05% SDS. Proteins were reduced with 5 mM TCEP for 30 min, and 100 µg of d0- or d8-ICAT was added to the protein extracts from raffinose- or galactose-grown cells, respectively. After 90 min of labeling at room temperature, the reactions were quenched by addition of β-mercaptoethanol to 10 mM, and combined. Each sample was diluted 10-fold with 20 mM Tris (pH 8.3), 0.01% SDS. Trypsin (10 µg) was added to digest proteins for 3 h at 37°C. The sample was diluted with an equal volume of buffer A (5 mM KH_2PO_4 (pH 3), 25% CH_3CN) and the pH was adjusted to 3 with dilute TFA. Either 100 µg (large-scale experiment) or 10 µg (small-scale experiment) of the combined protein digest was applied to a cation-exchange cartridge (Applied Biosystems, Foster City, CA) equilibrated in buffer A. The cartridge was washed with 2 ml buffer A, followed by 2 ml of buffer A + 40 mM KCl. Bound peptides were eluted with 600 µl buffer A + 600 mM KCl. Sample volume was reduced to 300 µl under reduced pressure, and 500 µl 2× PBS and 12 µl 1 M NH_4HCO_3 were added. Samples were passed over a monomeric avidin cartridge (Applied Biosystems) and washed with 2 ml 2× PBS, 1 ml 1× PBS, and 1 ml 50 mM NH_4HCO_3 with 20% methanol. Labeled peptides were eluted with 1 ml 0.4% TFA with 30% acetonitrile, dried under reduced pressure, and resuspended in 10 µl of 0.4% acetic acid with 5% acetonitrile. For either the large- or small-scale experiment, the same amount of sample was analyzed by the same µLC-MS/MS method as used in the solid-phase method.

Note: Supplementary information is available on the Nature Biotechnology *website.*

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