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ARTICLE

Affordable uniform isotope labeling with ^{2}H, ^{13}C and ^{15}N in insect cells

Agnieszka Sitarska[1] · Lukasz Skora[1] · Julia Klopp[1] · Susan Roest[1] · César Fernández[1] · Binesh Shrestha[1] · Alvar D. Gossert[1]

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Abstract For a wide range of proteins of high interest, the major obstacle for NMR studies is the lack of an affordable eukaryotic expression system for isotope labeling. Here, a simple and affordable protocol is presented to produce uniform labeled proteins in the most prevalent eukaryotic expression system for structural biology, namely S*podoptera frugiperda* insect cells. Incorporation levels of 80 % can be achieved for ^{15}N and ^{13}C with yields comparable to expression in full media. For ^{2}H,^{15}N and ^{2}H,^{13}C,^{15}N labeling, incorporation is only slightly lower with 75 and 73 %, respectively, and yields are typically twofold reduced. The media were optimized for isotope incorporation, reproducibility, simplicity and cost. High isotope incorporation levels for all labeling patterns are achieved by using labeled algal amino acid extracts and exploiting well-known biochemical pathways. The final formulation consists of just five commercially available components, at costs 12-fold lower than labeling media from vendors. The approach was applied to several cytosolic and secreted target proteins.

Keywords Eukaryotic cells · Insect cells · Protein expression · Isotope labeling · Uniform isotope labeling · NMR · Deuteration

Introduction

Current biological questions can often only be studied with proteins produced in eukaryotic expression systems. For structural biology, insect cells—typically Sf9 and Sf21 cells (Vaughn et al. 1977)—have become the eukaryotic expression host of choice. For NMR studies, however, isotope labeling has been limited to amino acid specific labeling (Brüggert et al. 2003; Strauss et al. 2003; Takahashi and Shimada 2009; Gossert et al. 2011; Gossert and Jahnke 2012) and only recently, more affordable protocols for uniform ^{15}N and ^{2}H,^{15}N labeling have become available (Strauss et al. 2005; Kofuku et al. 2014; Meola et al. 2014). Here we present a robust protocol for uniform labeling of ^{2}H, ^{13}C and ^{15}N in insect cells, which yields high isotope incorporation levels and at the same time is inexpensive and simple to use.

In order to obtain uniformly isotope labeled proteins in insect cells, all sources of amino acids in the growth medium need to be replaced with labeled ones. Since insect cell metabolism is limited, these sources are primarily pure amino acids added to the medium and yeast extract. Generally, an amino acid-free basal medium is ordered from vendors (in this work amino acid free Sf-900^{TM} III from Gibco LifeTechnologies, and amino acid free SF-4 from Bioconcept) and the desired amino acids are added. There are several strategies in order to replace unlabeled amino acids. Addition of all amino acids in their pure form with the desired isotope label typically results in prohibitive prices. A slightly less expensive alternative is to purchase ready made labeling media (e.g. Bioexpress-2000, CIL), which however still cost roughly 10,000 and 20,000 USD per liter for ^{15}N and ^{15}N,^{13}C labeling, respectively (Strauss et al. 2005). Additionally, these media are not available for ^{2}H,^{15}N or ^{2}H,^{13}C,^{15}N labeling. Another possibility is to use

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✉ Alvar D. Gossert
alvar.gossert@novartis.com

[1] Novartis Institutes for BioMedical Research, 4002 Basel, Switzerland

labeled yeast extracts to replace most of the amino acids (Egorova-Zachernyuk et al. 2009; Meola et al. 2014). This approach is more economical than the previous one, but requires the production of suitably labeled yeast, followed by autolysis and purification of the extract, increasing both, the required workload and the final cost of the medium. In this communication, isotope labeled algal extracts are used to supply most of the amino acids in an insect cell medium (Hansen et al. 1992; Kofuku et al. 2014). Algal extracts are fundamentally less expensive to produce than yeast extracts, as algae are grown on the simplest isotope labeled substrates i.e. $^{15}NH_3$, $^{13}CO_2$ and $^{2}H_2O$, while yeast require more expensive ^{13}C- and $^{2}H,^{13}C$-labeled carbohydrates (typically glucose). Additionally, algal hydrolysates have a very high amino acid content of >60 %, as compared to 30 % before and 50 % after enrichment for yeast extract (Egorova-Zachernyuk et al. 2009; Meola et al. 2014). Finally, algal extracts from *Spirulina* with all relevant isotopic labeling patterns (^{15}N; $^{2}H,^{15}N$; ^{13}C; $^{13}C,^{15}N$; $^{2}H,^{13}C,^{15}N$) are available as commercial products (ISOGRO® from Sigma-Aldrich, Celtone® from CIL).

Materials and methods

Expression

A detailed protocol covering medium preparation and the entire expression procedure is available as supporting information. In brief, a stock of Sf9 insect cells (Gibco LifeTechnologies) was diluted in full medium to 1.5×10^6 cells/mL. After 8 h of incubation in the medium, cells were infected with baculovirus harbouring the target gene DNA. After additional 16 h of incubation at 27 °C, cells were gently centrifuged, washed with amino acid free medium and transferred into labeling medium. After 72–96 h of incubation post infection, cells were harvested by centrifugation (1500*g*, 15 min, RT). For expression of Abl kinase, 20 µM imatinib was added for stabilization of the protein. Cell count and other parameters of both the stock and expression cultures were monitored daily on a Vi-CELL cell viability analyzer (Beckmann Coulter).

Metabolic inhibitors

Metabolic inhibitors L-cycloserine, aminooxyacetic acid hemihydrochloride (AOAA) and bithionol (all from Sigma-Aldrich) were prepared as 50 mM stock solutions in water for cycloserine and in DMSO for the latter two. Inhibitors were added at final concentrations of 50 µM for cycloserine and AOAA, and 10 µM for bithionol. The final concentration of DMSO in the insect cell culture was kept below 0.5 %, which we determined to be the toxicity limit.

Purification of proteins

All proteins were produced with N- or C-terminal His_6-tag. Cells were lyzed by sonication. After centrifugation, the soluble fraction was purified by Ni–NTA (1 mL Ni resin cartridge, QIAGEN).

Protein analytics and determination of isotope incorporation

The concentration of the expressed protein in the eluate was determined by HPLC-UV_{215} (Agilent Technologies 1200 series). Isotope incorporation was determined by comparing the molecular weight of unlabeled and labeled proteins. Mass spectrometry data were obtained on an Acquity ultra-high pressure liquid chromatography station using a Poros R1H 1 × 15 mm column equilibrated at 75 °C coupled to a Xevo G2-S QT mass spectrometer with electrospray ionization (Waters).

NMR spectroscopy

All spectra were recorded on a 600 MHz Bruker Avance III spectrometer equipped with a TCI cryoprobe with magnetic field gradients. All measurements were carried out at 23 °C.

Results

Uniform labeling with ^{15}N: replacing amino acids missing in algal hydrolysates

Our approach is to add the maximum amount of isotope labeled amino acids in order to obtain highest possible isotope incorporation into the expressed proteins and to minimize amino acid metabolism of the cells, which may lead to isotope dilution. The amount of algal extract that can be added to an insect cell medium is limited by the maximal osmolarity of the final medium tolerated by insect cells (320 mOsm/kg). We found that cell growth and viability was not negatively affected by up to 10–12 g of ISOGRO® per liter. Therefore, 10 g/L of ISOGRO® were used in the following experiments.

Unfortunately, algal hydrolysates lack several amino acids, namely asparagine, glutamine, cysteine and tryptophan, due to the protein hydrolysis step in the production process. These amino acids are usually present in insect cell media at concentrations of 1–2 g/L for Asn and Gln and 200 mg/L for Cys and Trp (O'Reilly et al. 1992). Adding such amounts of these amino acids in labeled form to an algal hydrolysate-based medium would significantly increase the cost. Therefore, we aimed at either reducing

the amount of these amino acids or replacing them with less expensive substrates, as discussed for each amino acid in the following (Hansen et al. 1992; Meola et al. 2014). Tryptophan is an essential amino acid, and therefore needed to be supplemented, but its concentration could be lowered tenfold to 20 mg/L. Even at this low starting concentration after a typical expression over 72 h, about 4 mg/L remained in the medium, as revealed by NMR-analysis of the growth medium. Cysteine can be synthesized by insect cells in several steps from methionine and serine (Doverskog et al. 1998), which are present in the algal lyate-based medium at concentrations of 300 and 400 mg/L, respectively. Glutamine and asparagine are non-essential amino acids and can be synthesized by insect cells. If ammonia is supplied, glutamine is synthesized from glutamate by the enzyme glutamine synthase (Fig. 1, enzyme 2). Another enzyme, asparagine synthase (Fig. 1, enzyme 1), will in turn use the side chain nitrogen of glutamine to synthesize asparagine from aspartate. Therefore, $^{15}NH_4Cl$ was added to the medium. We have determined the highest concentration without adverse effects on cell growth and viability to be 250 mg/L (5 mM), in line with several studies in literature (Hansen et al. 1992; Öhman et al. 1996; Meola et al. 2014).

The final formulation for uniform ^{15}N labeling consists therefore of an amino acid-free basal medium (Sf-900 III or SF-4), 10 g/L of ^{15}N-labeled ISOGRO®, 250 mg/L of $^{15}NH_4Cl$ and 20 mg/L of $^{15}N_2$-Tryptophan (Fig. 2). In order to assess the ^{15}N labeling efficiency, Abl kinase was expressed in Sf9 cells using this medium. As shown in Fig. 3, all amino acid types were labeled with ^{15}N, including those not present in the medium i.e. cysteine, asparagine and glutamine. The overall ^{15}N-incorporation was 83 %, as assessed by mass spectrometry, and it was

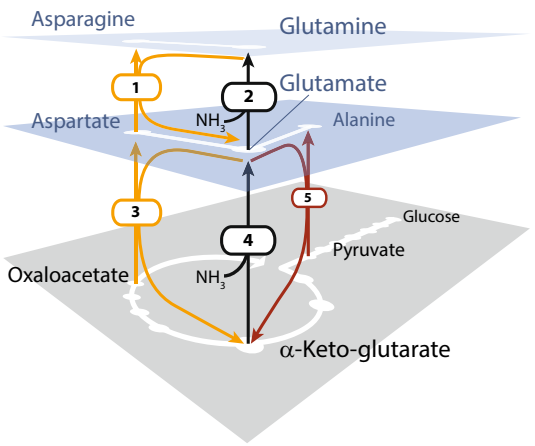

Fig. 1 Metabolic pathways involved in synthesis of selected N_1 and N_2 amino acids in insect cells. The *bottom plane* (*gray*) depicts glycolysis and the citric acid cycle; selected metabolites are indicated with their names. The *middle level* (*blue*) shows amino acids that are synthesized in a one-step reaction from the respective carbohydrates by addition of one nitrogen. The *top level* (*light blue*) shows amino acids with yet an additional nitrogen. Enzymatic reactions are marked with *arrows* and the enzymes are enumerated as follows: *1* asparagine synthetase, *2* glutamine synthetase, *3* aspartate transaminase, *4* glutamate dehydrogenase, *5* alanine transaminase

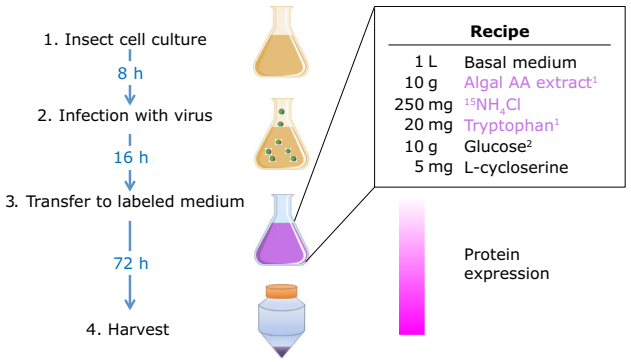

Fig. 2 Pictorial summary of the protocol for uniform labeling in Sf9 insect cells. On the *left*, the sequence of events during the expression culture is depicted. Protein expression only starts 24–48 h after infection with virus, as indicated by the *magenta* gradient on the *lower right*. On the *upper right side*, the recipe of the *medium* is shown, where isotope labeled components are shown in *magenta*. (*1*) The algal extract and tryptophan need to be labeled with the desired pattern, e.g. ^{15}N only, or $^{2}H,^{13}C,^{15}N$ etc. (*2*) Glucose doesn't need to be labeled for most applications (see text), only if triple resonance experiments are intended, glucose is replaced by 5 g/L of ^{13}C-glucose

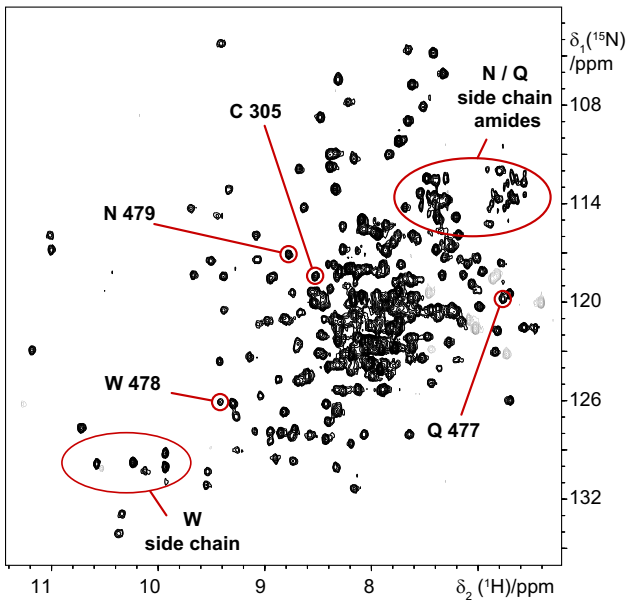

Fig. 3 Uniform ^{15}N labeling of all amino acid types. ($^{15}N,^{1}H$)-TROSY spectrum of u-^{15}N-labeled Abl (80 % incorporation) produced in Sf9 insect cells, in complex with the inhibitor imatinib (Vajpai et al. 2008). All expected correlation signals are seen, including backbone and side chain signals of the amino acids not present in the algal lysate: asparagine, cysteine, glutamine and tryptophan. For each of these amino acid types, at least one signal is indicated by red circles. In order to also detect NH_2 moieties in the TROSY experiment, the INEPT delay was shortened to 2.1 ms

uniform among all amino acids, e.g. no amino acid type yielded significantly lower signal intensity in the NMR spectrum.

However, infection of insect cells with baculovirus—the vehicle used for transfection of the cells with DNA of the target protein—is strongly hampered in this medium. That is due to inhibition of viral entry from the endosomes into the cytoplasm of insect cells by NH_4Cl (Hefferon et al. 1999; Dong et al. 2010). Therefore, cells need to be infected with baculovirus before they are transferred into the labeling medium. Infecting cells in non-labeled full medium has only a marginal effect on isotope incorporation, since target proteins are under control of the polyhedrin promoter, which is only activated in the late stage in the virus life cycle, typically 24–48 h post infection. Although virus entry should be completed after 1 h, in our hands expression levels were restored to comparable levels as in non-labeled medium only if cells were incubated with the virus for 16 h in full medium (Meola et al. 2014). After this time, the cells were gently centrifuged (300*g*), washed with amino acid-free medium, and transferred to the labeling medium. The drop of ^{15}N incorporation level from 83 to 80 % resulting from this procedure is acceptable considering the improved protein yields. The final protocol (Fig. 2 and supplementary material) is fairly simple and allows for inexpensive uniform labeling of proteins with ^{15}N in insect cells (Fig. 3).

Uniform labeling with ^{13}C: controlling carbohydrate incorporation into amino acids

The same protocol as described for ^{15}N can be applied for labeling of proteins with ^{13}C and ^{2}H with minor modifications. While in case of ^{15}N labeling, glucose metabolism can be safely ignored, it is relevant for incorporation of ^{13}C into some amino acids. Alanine, glutamate and aspartate are synthesized in one-step enzymatic reactions from metabolites of glucose, namely pyruvate, α-keto-glutarate and oxaloacetate, respectively (Fig. 1). Therefore, any unlabeled glucose present in the medium will be readily incorporated into these three amino acids. Moreover, since asparagine and glutamine are synthesized exclusively from aspartate and glutamate in our setting, also these will inherit unlabeled carbons. When using the above protocol with ^{13}C,^{15}N-labeled ISOGRO® and unlabeled glucose, an overall isotope incorporation of 75 % was achieved, as determined by mass spectrometry. Labeling efficiency was further increased to 81 % by adding ^{13}C-labeled glucose at a concentration of 5 and 10 g/L to a SF-4 and Sf-900 basal medium without glucose, respectively. However, as this increases the costs of the media considerably, we looked for a more economical solution in order to obtain acceptable ^{13}C labeling levels of Ala, Glx and Asx. An alternative solution is important especially in light of prospective deuteration, as addition of ^{2}H,^{13}C glucose to insect cell media results in arrested cell growth and no protein expression.

In order to quantify incorporation of glucose into amino acids, ^{13}C-glucose was added to an otherwise non-labeled medium. In this experiment, only 10–20 % of Asx and Glx were derived from glucose, while nearly 80 % of Ala were synthesized from it (Strauss et al. 2005). That most of the alanine is actively synthesized by insect cells is also evident when analyzing the changes in amino acid content of media during cell culture. The NMR signal intensities of amino acids decrease over time as they are consumed by the cells. Alanine represents a striking exception: its concentration increases about fourfold over the typical 72-h expression period. If unlabeled glucose is used for production of ^{13}C-labeled proteins, the signals of methyl groups of alanine of such proteins are fivefold weaker than other methyl groups in an (^{13}C,^{1}H)-HSQC.

For routine NMR studies based on HSQC spectra, our approach is to economize by using non-labeled glucose and accepting weakly lowered ^{13}C incorporation rates of aspartate and glutamate, but to inhibit the enzyme alanine transaminase that converts unlabeled pyruvate into alanine (Fig. 1, enzyme 5). L-Cycloserine, a known non-toxic

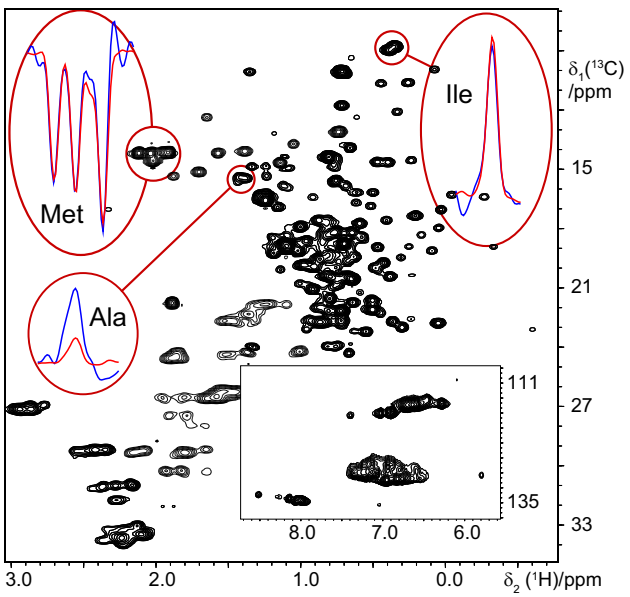

Fig. 4 Uniform ^{13}C labeling of all methyl groups including alanine. (^{13}C,^{1}H)-ct-HSQC of u-^{13}C,^{15}N-labeled Abl (76 % incorporation) produced in Sf9 insect cells. 1D traces show signals of selected amino acid types (scaled according to Met and Ile signals) after expression using unlabeled glucose with (*blue*) or without (*red*) 50 µM L-cycloserine. Alanine signal intensity can be fourfold increased by this method and a complete methyl spectrum can be obtained in an affordable way. In the excerpt on the *lower right* the aromatic region of a conventional (^{13}C,^{1}H)-HSQC is shown, demonstrating labeling of aromatic side chains

inhibitor of this enzyme (Wong et al. 1973), was added to a final concentration of 50 μM. With the addition of cycloserine complete methyl spectra of excellent quality could be obtained (Fig. 4). For $^{13}C,^{15}N$ labeling the overall isotope incorporation is 76 % and all amino acids are labeled at an acceptable level. It should be noted, that although the overall incorporation is 76 %, each individual labeled amino acid, except for Asx and Glx, has a ^{13}C incorporation level of 98 %. This is because fully labeled amino acids from the 98 %-labeled algal extract are integrated into proteins as a whole, without being metabolized. The lower overall incorporation is due to integration of fully unlabeled amino acids present in the cells, either from earlier growth media, or from degradation of unlabeled proteins. As a consequence, in NMR experiments intra-residual magnetization transfer steps are very efficient (98 %) and inter-residual ones have a lower efficiency (76 %). In an HNCA for example, the intra-residual H–N–CA pathway will yield an overall transfer efficiency of 94 % (0.98^3) for the approximately 76 % labeled amino acids in a protein. The inter-residual H–N–CA_{-1} pathway, however, will be 73 % efficient (0.98 × 0.98 × 0.76) because only 76 % of neighboring residues will statistically be labeled. That's why we suggest using labeled glucose for experiments involving inter-residual magnetization transfer, to increase transfer efficiency to close to 80 %.

Labeling of ^2H: minimizing amino acid metabolism

It is straightforward to extend the protocol to labeling patterns including deuterium (Fig. 5). For $^2H,^{15}N$ and $^2H,^{13}C,^{15}N$ triple labeling overall incorporation levels were 75 and 73 %, respectively, which are the highest reported so far (Kofuku et al. 2014). High amounts of algal hydrolysate in the medium ensure high isotope incorporation and lowered metabolism. However, protein expression yields are affected by deuterium labeling. We typically obtain 30–60 % of the protein amounts compared to expression in non-labeled medium (Table 1).

Deuterium incorporation depends on the same biochemical pathways as discussed for carbon incorporation above. Consequently, since non-deuterated glucose is used, approximately 30–40 % of Asp, Asn, Glu and Gln will have fully protonated carbon chains (20 % from unspecific background and 10–20 % from biosynthesis). Additionally, insect cell metabolism will lead to protonation of the alpha positions of several deuterated amino acids. From the analysis of an HNCA spectrum of $^2H,^{13}C,^{15}N$-labeled Abl kinase, at least partial protonation of the alpha position was seen for Glx (most severe), Asx and Gly. For all other amino acids no signs of back-protonation were detected. Protonation of the alpha positions in Asx and Glx could be explained with the reactions 3, 4 and 5 (Fig. 1), in which the alpha carbons become protonated. These reactions may also explain the lower yields obtained with deuterated amino acids. This can be rationalized taking glutamate as an example: In reactions 3, 5 and 4 (reversed) the amino acid will be transformed into deuterated α-keto-glutarate (Fig. 1). This may exhibit an effect on the citric acid cycle, slowing down several enzymes sevenfold due to the deuterium isotope effect. Cell metabolism may in general be hampered if the kinetics of the enzymes in the central citric acid cycle are not synchronized anymore. Ideally, by inhibiting enzymes 3–5 (Fig. 1), alpha-protonation could be suppressed and the yields restored. To this end the following inhibitors were tested: Aminooxyacetic acid (AOAA) for aspartate transaminase, bithionol for glutamate dehydrogenase and the previously discussed L-cycloserine for alanine transaminase (Wong et al. 1973; Rej 1977; Li et al. 2009). Bithionol could not be used due to its toxicity to the cells

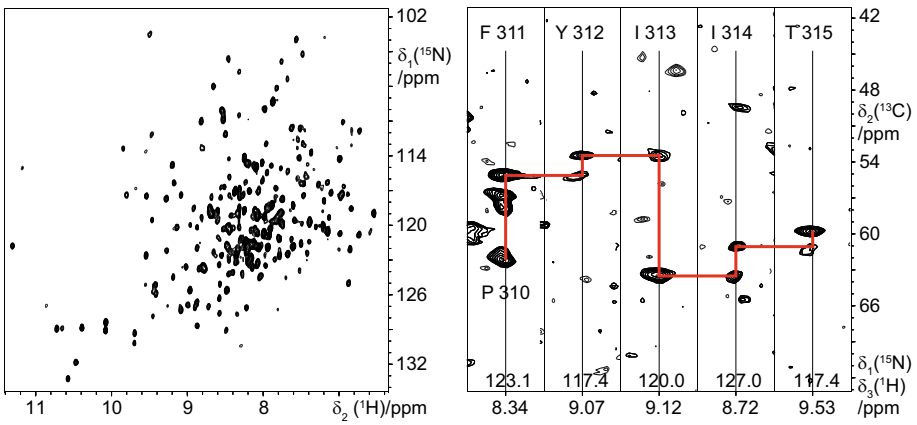

Fig. 5 Impact of deuteration on spectral quality. On the *left*, a ($^{15}N,^1H$)-TROSY spectrum of $^2H,^{15}N$-labeled Abl (76 % incorporation) produced in insect cells shows a significant improvement in comparison with Fig. 3. On the *right*, strips of a 3D-HNCA experiment showing sequential connectivities to the biologically important "gatekeeper" residue T315; the spectrum was recorded in only 16 h on a sample of 400 μM $^2H,^{13}C,^{15}N$-labeled Abl

Table 1 Application to different types of human proteins

Protein	Labeling pattern	Yield[a] (mg/L)	Incorporation[b] (%)
Cytosolic protein[c] 45 kDa	$^{13}C,^{15}N$	21.6	72[d]
	$^{2}H,^{15}N$	12.0	67[d]
Membrane protein 18 kDa	$^{13}C,^{15}N$	4.5	77
	$^{2}H,^{15}N$	1.8	73
Secreted protein 18 kDa	$^{13}C,^{15}N$	8.4	–[e]

[a] Protein yield determined by HPLC-UV_{215}

[b] Isotope incorporation level as determined by mass spectrometry

[c] Due to trade secrets, the names of proteins cannot be disclosed

[d] To further simplify the protocol, washing of cells post-infection with amino acid free medium was omitted, leading to reduction of incorporation by approximately 5 %

[e] Due to inhomogeneous glycosylation, mass determination was not possible for this protein

even at 10 μM concentration, and the combination of 100 μM AOAA with 50 μM cycloserine did not improve incorporation nor yields significantly over cycloserine alone. Therefore, either more effective inhibitors are needed or enzymatic pathways that were not considered here are responsible for alpha-protonation and lowered yields.

Summary

We have established a simple and economic protocol for uniform isotope labeling in insect cells, which is readily implemented since all ingredients of the media are commercially available. For all labeling patterns described, incorporation levels are the highest reported so far for non-commercial media (Meola et al. 2014; Kofuku et al. 2014). This is a consequence of the high amount of labeled amino acids that is added in our protocol, which is possible due to the affordable price of algal amino acid extracts. The 20 % of unlabeled amino acids originate from intracellular pools of the insect cells, and incorporation could be increased by several passages in labeling medium. However, this would not be economical, and the medium was not designed to sustain cell growth much beyond the typical 72–96 h used for expression.

^{13}C incorporation of 76 % is achieved using non-labeled glucose, and the strategy using L-cycloserine to control alanine un-labeling yields excellent results at lower costs. This approach is ideal for functional studies relying on complete methyl or aromatic ($^{13}C,^{1}H$)-HSQCs (Fig. 4). In case of triple resonance experiments for backbone assignments, it may be worth investing into ^{13}C-labeled glucose in order to obtain comparable signal intensities for carbohydrate-derived amino acids as for all other amino acids.

For ^{2}H labeling, incorporation of 76 % was obtained, leading to remarkable improvements in spectral quality (Fig. 5). Unfortunately, introduction of protonated carbohydrates into the side chains of Asp, Asn, Glu and Gln, as well as partial back-protonation of alpha positions of the same amino acids and Gly, result in an inhomogeneous deuteration pattern, which is a limitation in triple resonance experiments of large proteins. Still, the highest incorporation and most homogenous deuteration was achieved compared to other methods. This is due to the use of inhibitors and the high amino acid content of our media, which probably leads to reduced amino acid metabolism and product inhibition of metabolic enzymes. It should be noted that an additional advantage of this method is complete protonation of amides, since cells are grown in H_2O and not in D_2O as in deuteration protocols for *E. coli*. Therefore no partial unfolding steps are needed to re-protonate the protein. Further it is conceivable to use methyl-protonated, else deuterated algal extracts in this method, in order to produce selectively protonated proteins samples for studying large proteins and protein complexes (Linser et al. 2014).

These protocols have enabled NMR studies of several proteins that could only be expressed in eukaryotic systems in our laboratories (Table 1). The media are economic (800 USD/L for ^{15}N labeling; 1500 USD/L for $^{13}C,^{15}N$ and $^{2}H,^{15}N$; 3500 USD/L for $^{2}H,^{13}C,^{15}N$), easy to prepare (basal medium plus four ingredients) and all ingredients are readily available. Due to these three facts, we expect that these protocols will be readily reproducible in other laboratories and enable NMR studies based on uniform labeling like e.g. un-labeling approaches and resonance assignments as well as functional and structural studies of eukaryotic proteins.

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Conflict of interest The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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