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Communication

Efficient uniform isotope labeling of Abl kinase expressed in Baculovirus-infected insect cells

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Abstract

This report shows for the first time the efficient uniform isotope labeling of a recombinant protein expressed using Baculovirus-infected insect cells. The recent availability of suitable media for ^{15}N- and ^{13}C/^{15}N-labeling in insect cells, the high expression of Abl kinase in these labeling media and a suitable labeling protocol made it possible to obtain a ^{1}H–^{15}N-HSQC spectrum for the catalytic domain of Abl kinase of good quality and with label incorporation rates >90%. The presented isotope labeling method should be applicable also to further proteins where successful expression is restricted to the Baculovirus expression system.

Uniform isotope labeling of recombinant (rec.) proteins represents an important tool for the application of NMR methods to study the structure and dynamics of proteins alone or with interacting ligands. The great majority of the uniformly isotope labeled proteins reported in the literature so far have been expressed in *E. coli*, where efficient methods for ^{15}N-, ^{13}C/^{15}N- and ^{2}H/^{13}C/^{15}N-labeling and subsequent analysis by NMR have been described (e.g. Muchmore et al., 1989; Bax, 1994; Venters et al., 1995). However, complex proteins – many of these being important drug targets, such as kinases – can often not be expressed with *E. coli* in an active, correctly folded and post-translationally modified form and require eukaryotic host cells for successful expression. For mammalian cell cultures, a method for uniform isotope labeling of rec. proteins has been described (Hansen et al., 1992), but it was not applied generally. CHO cells were used to express TGF-β1 labeled uniformly and partly with ^{15}N and ^{13}C, leading to 96% resonance assignments in the ^{1}H–^{15}N-HSQC spectrum (Archer et al., 1993). More recently, the yeast *Pichia pastoris* was employed as expression host for uniform isotope labeling of rec. proteins (Wood and Komives, 1999; van den Burg, 2001). For one of the presently most frequently used eukaryotic expression system, the Baculovirus expression system (BVES), no reports on uniform isotope labeling of proteins have so far been published. The BVES is based on the infection of insect cells with rec. Baculovirus (BV) carrying the gene of interest and subsequent expression of the corresponding rec. protein by the insect cells (O'Reilly et al., 1994). Amino acid-type selective isotope labeling of proteins has been successfully performed with the BVES (Brüggert et al., 2003; Strauss et al., 2003).

In this communication, we report on a method for successful uniform ^{15}N- and ^{13}C/^{15}N-labeling of the catalytic domain of Abl kinase (Abl) expressed in BV-infected insect cells. We have chosen Abl as model protein for this study as it can be expressed correctly folded in sufficient amounts in BV-infected insect cells (Strauss et al., 2003)

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whereas expression in *E. coli* is poor. Furthermore Abl is a tyrosine kinase of great pharmaceutical interest, as it is the target of the anti-leukemia drug STI571 (Glivec®) (Fabbro et al., 2002). Amino acid-type selective isotope labeling (Strauss et al., 2003) and resonance assignment by spin labeling (Cutting et al., 2004) have been described for Abl. The crystal structure of Abl complexed to STI571 is known (Nagar et al., 2002).

For this study, the same construct of human Abl, isoform 1A was chosen as used and described earlier (Strauss et al., 2003). The expressed protein is constituted as 6His-tag/TEV-cleavage site/GAMDP/Abl(S^{229}–S^{500}), has 299 amino acids and a molecular weight of 34893.8 Da. The TEV-protease – processed form used for NMR and MS analysis is constituted of 277 amino acids with the sequence S^{229}–S^{500} of the catalytic domain of Abl kinase preceded by five amino acids (GAMDP) from the tag and has a molecular weight of 32014.7 Da. Expression in labeling medium was also tested for another protein tyrosine kinase, human VEGFR-2 kinase (KDR). A construct of the catalytic domain of KDR was employed in which 50 amino acids of the kinase insert domain had been deleted (M^{806}–K^{941}/Δ50/P^{992}–D^{1171}-thrombin-cleavage site-6His-tag, MW 37954 Da).

In general, methods for culturing insect cells, production of and infection with rec. Baculovirus and expression of rec. proteins were used as described by O'Reilly et al. (1994). Generation and amplification of the rec. BV for Abl expression was performed as described earlier (Strauss et al., 2003). The employed rec. BV suspensions were generated by the Bac-to-Bac transfection system (Invitrogen) for Abl and by the BaculoGold transfection system (Pharmingen) for KDR as suggested by the manufacturers. The resulting rec. BV were amplified in three rounds from a single viral plaque to a viral suspension in medium TC100 (Invitrogen) + 10% FCS (fetal calf serum; Invitrogen) of 2.25×10^8 pfu/ml (for Abl), determined by plaque assay and was stored at 4 °C in the dark. Sf9 cells were from ATCC strain collection. For expression cultures, a subline from Sf9 adapted to growth in suspension in serum-free medium SF900 II (Invitrogen) was used.

To perform isotope labeling of Abl at the liter-scale, the following protocol was employed: Sf9 cells adapted to SF900 II medium were precultured at 27 °C and 90 rpm for 3 days in 100 ml medium SF900 II in a 500 ml-Erlenmeyer flask, and 5×10 ml of this preculture were used to inoculate the main cultures of typically 5×100 ml SF900 II medium in 500 ml-Erlenmeyer flasks. After ca. 3 days at 27 °C and 90 rpm, when cells have reached a density of ca. 4×10^6 cells/ml, suitable volumes of these cultures for a final cell density of 1.5×10^6 cells/ml were transferred to 50 ml-plastic tubes and were centrifuged at 400*g* at room temperature (rt) for 10 min. The supernatants were discarded as completely as possible, cells were resuspended in 5×30 ml labeling medium and transferred to 5×500 ml-Erlenmeyer flasks. All five cultures were filled up with labeling medium to 100 ml resulting in cell densities of 1.5×10^6 cells/ml, and 1.3 ml of rec. BV suspension of a titer of 2.27×10^8 pfu/ml was added to each culture, corresponding to a MOI = 2. In order to improve the stability and to reduce the phosphorylation of Abl during expression, STI571 was added to the cultures at the time of infection to a final concentration of 16–20 μM (Strauss et al., 2003). Cultures were further incubated for 3 days post infection (p.i.) at 27 °C and 90 rpm. Abl-expressing cells were harvested by two subsequent centrifugations at 400*g* at rt for 20 min, first in 1 l-centrifuge bottles and then after having taken up the pelleted cells in 20 ml PBS, pH 6.2 with a protease inhibitor mix (Complete™, Roche) in 50 ml-plastic tubes. Cell pellets were stored frozen at −80 °C prior to extraction.

For testing media and culture conditions, precultures and main cultures were also carried out as given above for the liter-scale cultures performing in general a transfer of cells from growth to labeling medium, but cells were resuspended at a density of 1.5×10^6 cells/ml in 50 ml labeling or control medium in 200 ml-Erlenmeyer flasks before addition of a corresponding amount of rec. BV for MOI = 2 and further culturing for 3 days p.i. at 27 °C and 90 rpm. In case of direct growth and expression in labeling medium BE2000-U, centrifuged cells from 5 ml precultures were taken up in 50 ml BE2000-U medium and cultured and infected as given above. For KDR expression, 10 μM (final conc.) of the inhibitor PTK787 (Bold et al., 2002) was added to the cultures at the time of infection with rec. BV (MOI = 1).

SF900 II (Invitrogen, #10902-088) and EX-CELL 420 (JRH, #14420-1000M) were used as

expression media for comparision. The following labeling media from CIL, were used: unlabeled medium, 'BioExpress-2000 (Insect Cell) (Unlabeled)' (CIL, #CGM-2000-U), called here BE2000-U; ^{15}N-labeling medium, 'BioExpress-2000 (Insect Cell) (U–^{15}N, 98%)' (CIL, #CGM-2000-N), called here BE2000-N; ^{13}C/^{15}N-labeling medium, 'BioExpress-2000 (Insect Cell) (U–^{13}C, 92–95%, U–^{15}N, 92–95%)' (CIL, #CGM-2000-CN), called here BE2000-CN. The following two labeling media derived from BioExpress-2000 were obtained custom made from CIL: 'BioExpress-2000 (Insect Cell) (^{13}C/^{15}N-labeled for L-methionine, L-leucine, L-isoleucine and L-tyrosine)', called here BE2000-CN-MLIY and 'BioExpress-2000 (Insect Cell) (^{13}C-carbohydrates)', called here BE2000–^{13}C-carboh..

At harvest time, 1 ml-samples for SDS-PAGE analysis and 30 ml-samples for cell fresh weight determination were taken and centrifuged at 300*g* at rt for 10 min. Cell pellets from the 1 ml-samples were taken up in 1 ml 1 × SDS-PAGE buffer and an amount of total lysate equivalent to 0.2 mg fresh weight cells was applied on the gels for SDS-PAGE analysis to estimate Abl expression. Cell density and viability was determined by counting viable and dead cells in a hemocytometer. Cell diameter was measured by a Cedex cell counter. Total Abl expression was estimated by gel-densitometry measuring the intensity of the Abl-band on a Coomassie-stained gel in relation to the total protein content measured by the Bradford method (BIO-RAD).

For isolation of labeled Abl, cell pellets were lysed by mild sonication in 50 mM Na-phosphate, 300 mM NaCl, 10% by vol. glycerol, 10 mM 2-mercaptoethanol, pH 8, supplemented with a protease inhibitor cocktail and with 10–20 μM STI571. After clarification by centrifugation and filtration, the lysate was applied to a column of Ni-NTA superflow resin (Quiagen). After washing with 10 and 15 mM imidazole, the bound 6His-Abl/STI571 complex was eluted with 100 mM imidazole in Tris-buffer, 10% by vol. glycerol, 100 mM NaCl, pH 8 and – after addition of EDTA and DTT (2 mM each) – was incubated with AcTEV-protease (Invitrogen) overnight to remove the His-tag. The cleavage reaction was purified on a Mono Q anion-exchange column (HR10/10, Amersham Biosciences) using a gradient from 50 to 300 mM NaCl in 20 mM Tris, 5% by vol. glycerol, 2 mM DTT buffer, pH 8. The main peak consisting of unphosphorylated Abl/STI571 was concentrated and applied to a Superdex 75 size exclusion column (HiLoad 16/60, Amersham Biosciences) equilibrated in 20 mM Bis–Tris, 100 mM NaCl, 2 mM EDTA, 3 mM DTT, pH 6.5. Fractions containing pure Abl/STI571 complex were combined, and concentrated by ultrafiltration. All purification steps were performed at 4 °C and were analyzed by reversed phase HPLC to determine the concentrations of Abl and STI571; the samples used for NMR studies showed a molar ratio of about 1:1. The amount of label incorporated into the different Abl samples was determined by LC-MS (electrospray ionization) analysis of the unphosphorylated form of Abl obtained by Mono Q separation. For NMR analysis, Abl was concentrated to about 14 mg/ml (0.4 mM, ^{15}N- and ^{13}C-/^{15}N-MLIY-Abl) and to 4.7 mg/ml (0.14 mM, ^{13}C-/^{15}N-Abl). NMR spectra were acquired at 23 °C with a 600 MHz or a 800 MHz Bruker DRX spectrometer.

The results in Figure 1 and Table 1 show that the expression levels of Abl in labeling media of the BE2000 series are as high as in control media SF900 II or EX-CELL 420 and are thus sufficiently high for isolation of at least 50 mg Abl from 1 l culture (Table 2). Expression levels as high as 85 mg/l of isolated and soluble 6His-Abl could be reached in case of expression in ^{13}C/^{15}N-labeling medium BE2000-CN. The purified labeled Abl protein (complexed to STI571) was correctly folded and sufficiently stable for NMR analysis as confirmed by a ^{1}H–^{15}N-HSQC spectrum of good quality (Figure 3). Although the kinase domain of Abl is rather large (32 kDa), it is still suitable for NMR analysis. Other rec. proteins, as illustrated in Figure 1 for the example of KDR, are expressed in insect cells in unlabeled BE2000-U medium as high as in SF900 II (data for other proteins not shown), suggesting that BE2000 is also a suitable medium for successful isotope labeling of other rec. proteins by expression in insect cells.

A suitable labeling medium is a crucial factor for successful isotope labeling of rec. proteins in insect cells. Culture media for uniform ^{15}N-labeling of rec. proteins with insect cells suitable for getting a ^{1}H–^{15}N-HSQC spectrum have been commercially available for a few years (e.g. 'BioExpress-2000, ^{15}N-labeled, for insect cells', old formulation, from

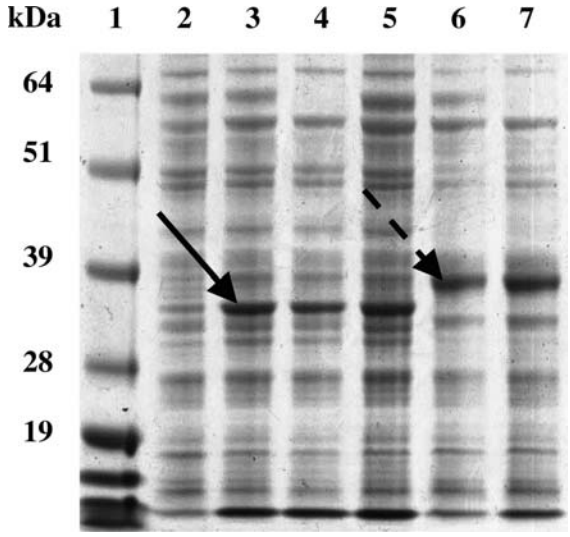

Figure 1. SDS-PAGE analysis by Coomassie blue staining of Abl or KDR expression by BV-infected Sf9 cells in 50 ml shake flask cultures expressing Abl or KDR in labeling or control media. Growth and expression in BE2000-U without medium change (lane 2) or growth in SF900 II and medium change to the labeling/expression medium (lanes 3–7). Expression of unlabeled Abl in BE2000-U (lanes 2 and 3), in SF900 II (lane 4) or of $^{13}C/^{15}N$-labeled Abl in BE2000-CN (lane 5). Expression of unlabeled KDR in BE2000-U (lane 6) and in SF900 II (lane 7). Molecular weight marker (Invitrogen, SeeBlue Plus2) with given size (lane 1). Full arrow points to Abl, dashed arrow points to KDR. Low Abl expression for a culture without medium change (lane 2) could be confirmed by a faint band on a Western blot treated with anti-Abl-ab (Oncogene, #OP19-100UG) (data not shown).

CIL). However no $^{1}H–^{15}N$-HSQC spectrum from a rec. protein expressed in insect cells has been published so far, probably at least partly due to the high cost for such a medium. For insect cells, as for other animal cells, all 20 (or at least the 10 essential) amino acids have to be present in labeled form in an expression medium for uniform isotope labeling and cannot be synthesized from ^{13}C-glucose and $^{15}NH_4^+$ (and D_2O) as in *E. coli*. Many of the 20 amino acids are expensive in the ^{15}N-labeled and even more so in the $^{13}C/^{15}N$-labeled form at the quantities required (100–2000 mg/l) for maximum protein expression. As for frequently used culture media for protein expression in insect cells such as SF900 II and EX-CELL 420, the source and the concentration of the amino acids in BE2000 media has not been disclosed to the public, but for some insect cell media used for expression, such as IPL-41, the composition is known (Weiss et al., 1981). For uniform ^{15}N-labeling, label dilution by unlabeled NH_4^+ can be excluded, as fresh insect cell media (as labeling media BE2000) do not contain NH_4^+.

We uniformly ^{15}N-labeled Abl with rec. BV-infected Sf9 cells using labeling medium BE2000-N and the herein described culture protocol and obtained 91.4% label incorporation rate (Table 2). A $^{1}H–^{15}N$-HSQC spectrum for ^{15}N-labeled Abl of good quality was obtained (data not shown), corresponding well to the one shown below for $^{13}C/^{15}N$-labeled Abl (Figure 3).

For uniform $^{13}C/^{15}N$-labeling, useful for resonance assignment in a $^{1}H–^{15}N$-HSQC spectrum, ideally a labeling medium for insect cells should contain all 20 amino acids in $^{13}C/^{15}N$-labeled form to such a high degree, that the label incorporation rate in total, but also for each single amino acid is at least 90%. Label dilution by unlabeled NH_4^+ can be excluded for the same reason as given above for uniform ^{15}N-labeling, but ^{13}C-label dilution by the carbohydrates serving as carbon source in an insect cell culture medium is possible. To investigate this possibility, Abl expression by BV-infected Sf9 cells was performed in a custom-made medium BE2000−^{13}C-carboh. in which all carbohydrates were uniform ^{13}C-labeled and the purified unphosphorylated Abl protein was subjected to LC-MS analysis. The observed increase in molecular mass of Abl can account for a ^{13}C-incorporation

Table 1. Cell density, viability, yield and diameter and Abl expression levels at 3 days post infection of BV-infected Sf9 cells cultivated after medium change in 50 ml shake flask cultures in labeling medium BE2000-U compared to standard expression media

Culture Medium	Cell density ($\times 10^6$ c/ml)	Viability (%)	Fresh weight (g/30 ml)	Cell diameter (μm)	Abl expression (mg/l)
BE2000-U	1.60 ± 0.09	93.3 ± 1.7	0.50 ± 0.01	24.0 ± 0.8	269 ± 20
SF900 II	1.74 ± 0.22	90.3 ± 1.4	0.51 ± 0.03	24.3 ± 1.1	286 ± 42
EX-CELL 420	1.60 ± 0.06	89.9 ± 1.4	0.56 ± 0.03	24.3 ± 1.6	216 ± 8

Abl expression is quantified by gel-densitometry and determining total protein content (see above). Mean values from three parallel cultures and the resulting standard deviations are given.

Table 2. Yield of isolated 6His-Abl protein, label incorporation rates and performed NMR studies from liter-scale productions of isotope-labeled Abl kinase expressed in rec. BV-infected Sf9 cells

Labeling type of Abl (Medium)	Isolated 6His-Abl protein[a]	Label incorporation rate (%)[b]	Performed NMR analysis
Unlabeled (BE2000-U)	26 mg/0.44 l (59 mg/l)	–	–
Uniform ^{15}N-labeling (BE2000-N)	16 mg/0.24 l (67 mg/l)	91.4	^{1}H–^{15}N-HSQC spectrum
U–^{13}C/^{15}N–Met/Leu/Ile/Tyr-labeling (BE2000-CN-MLIY)	54 mg/0.98 l (55 mg/l)	93.0	^{1}H–^{15}N-HSQC spectrum
^{13}C-partly labeling (BE2000–^{13}C-carboh.)	60 mg/0.89 l (67 mg/l)	9.2	–
Uniform ^{13}C/^{15}N-labeling (BE2000-CN)	40 mg/0.47 l (85 mg/l)	90.5	^{1}H–^{15}N-HSQC spectrum

[a]6His-Abl eluted from Ni-NTA column measured by HPLC given in mg per volume of the actually used cultures and in brackets calculated in mg/l. [b]Label incorporation rates are calculated as percentage of the observed to the theoretical mass increase in MS spectra of purified unphosphorylated Abl samples.

rate of 9.2% (Table 2). LC-MS analysis on the individual, dabsyl-chloride derivatized amino acids obtained from a hydrolyzate of such an Abl protein showed that ^{13}C-incorporation has occurred into the amino acids Ala, Glu/Gln and Asp/Asn with incorporation rates of about 77, 28 and 16%, respectively. ^{13}C-incorporation into the other amino acids was below the detection limits of the LC-MS analysis; for Trp a determination is not possible. Thus for reaching high ^{13}C/^{15}N-label incorporation rates, a labeling medium on the basis of BE2000 has to contain all carbohydrates serving as carbon source in a ^{13}C-labeled form; this is the case for BE2000-CN used here.

In the same way as for ^{15}N-labeling described above, Abl was expressed in ^{13}C/^{15}N-labeled form but using medium BE2000-CN for expression. Purified ^{13}C/^{15}N-labeled Abl (in complex with STI571) was subjected to LC-MS and NMR analysis. ^{13}C/^{15}N incorporation rate was estimated approximately 90.5% as shown by electrospray mass spectrometry (Figure 2). The majority of the amino acid types being hydrolyzed from ^{13}C/^{15}N–labeled Abl and converted to dabsyl-derivates shows in LC-MS analysis label incorporation rates exceeding 90%; only for Gly (81%), Arg (81%) and His (73%) the incorporation rates are somewhat lower. The resulting ^{1}H–^{15}N-HSQC spectrum, shown in Figure 3, is of good quality. The majority of the 277 amino acids of GAMDP-Abl(229–500) show up as clear and defined resonances in the ^{1}H–^{15}N-HSQC spectrum. Superimposed resonances of ^{15}N–Phe-, ^{15}N–Tyr- and ^{15}N–Val-labeling from amino acid-type selective ^{15}N-labeling of Abl (Strauss et al., 2003) match perfectly with resonances

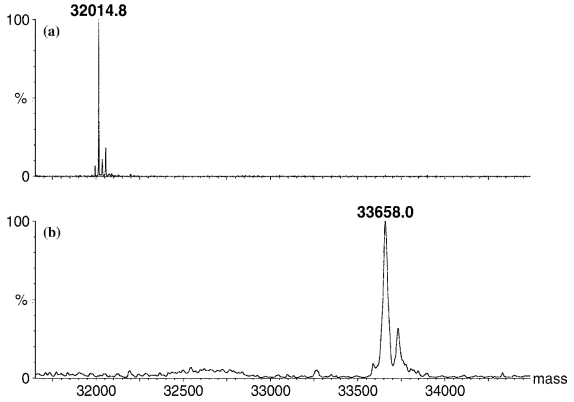

Figure 2. Deconvoluted ESI-MS of unlabeled (a) and ^{13}C/^{15}N-labeled Abl (b). X-axis displays molecular mass, y-axis displays relative intensities. The theoretical masses of the unlabeled Abl and ^{13}C/^{15}N-labeled Abl were 32014.7 and 33826.7 Da, respectively (1446 carbon and 366 nitrogen atoms). The measured masses of unlabeled Abl and ^{13}C/^{15}N-labeled Abl were 32014.8 and 33658.0 Da, respectively. The labeling efficiency (incorporation rate) was therefore estimated 90.5%.

from uniform ^{13}C/^{15}N-labeling. The uniformly ^{13}C/^{15}N-labeled Abl sample will now serve us for resonance assignment.

Two possible culture protocols are possible for uniform isotope labeling: either insect cells are grown and infected by rec. BV in the labeling medium or insect cells are pre-grown in an unlabeled growth/expression medium and transferred after centrifugation to the labeling medium prior to infection with rec. BV. The two possibilities were compared for Abl expression with unlabeled medium BE2000-U. As shown in Figure 1 and analogous to earlier observations

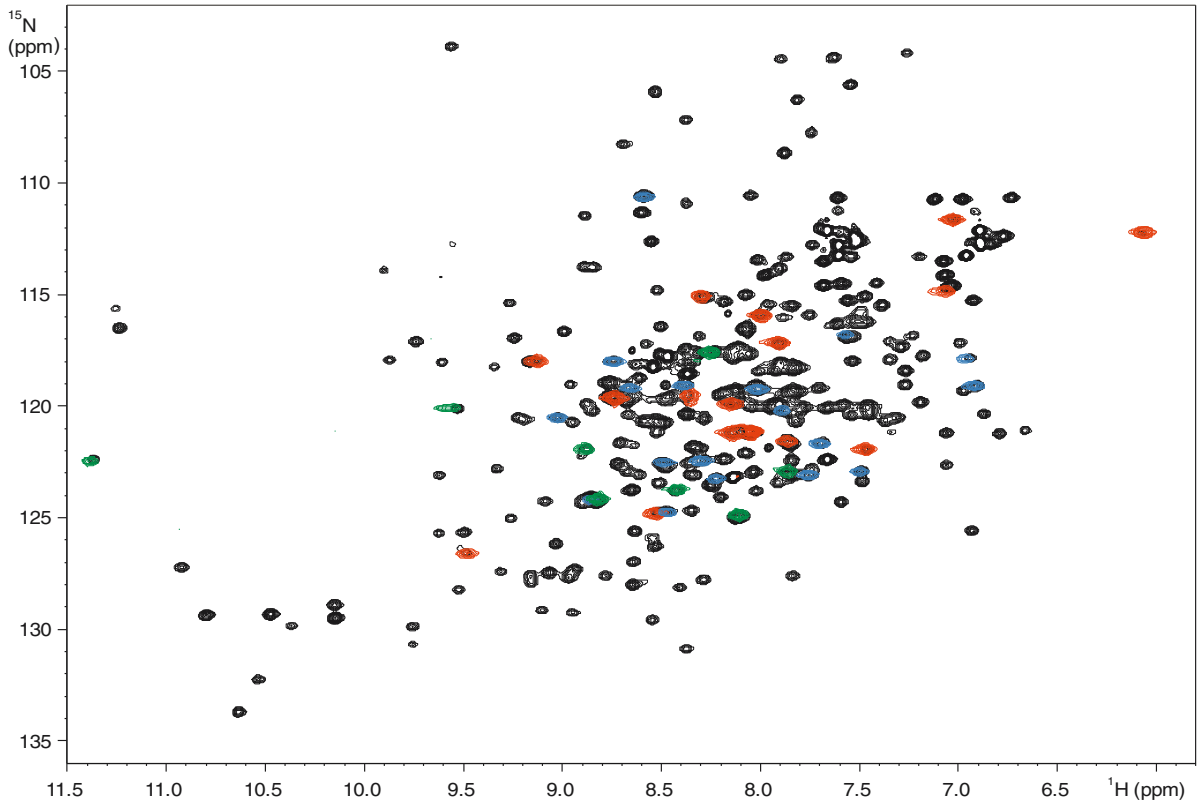

Figure 3. [^{15}N;^{1}H]-HSQC spectrum for uniformly ^{13}C/^{15}N-labeled Abl kinase (in black) expressed in BV-infected Sf9 cells, superimposed with the resonances from Abl kinase labeled with ^{15}N-Phe (in green), ^{15}N-Tyr (in red), ^{15}N-Val (in blue) (from Strauss et al., 2003). One resonance at 133.4 ppm (^{15}N)/12.6 ppm (^{1}H) lies outside the scales in the figure.

with amino acid-selective labeling (Strauss et al., 2003), Abl expression and cellular yield are distinctly higher when cells are transferred from an expression medium to a labeling medium as compared to a labeling protocol without medium change. Essentially the same culture protocol with medium change was thus chosen for uniform isotope labeling of Abl as applied for amino acid-type selective labeling (Strauss et al., 2003), except that here Sf9 cells were grown in medium SF900 II (and not in medium SF-4) to a higher cell density before medium change, Sf9 cells were grown in several 100 ml-cultures in 500 ml-Erlenmeyer flasks and the labeling medium was labeled BE2000 medium. The carry over of unlabeled amino acids in this culture protocol (from the cell-internal amino acid pool and from added medium with rec. BV) leading to label dilution is obviously negligible, as in the cases of ^{15}N-, ^{13}C/^{15}N- and ^{13}C/^{15}N-MLIY-labeling where the labeling media are labeled >90%, incorporation rates of ≥90% could be achieved (Table 2; Strauss et al., 2003).

High expression of the model protein Abl under labeling conditions, suitable labeling media from the BioExpress-2000 (Insect Cell) series from CIL and an optimized labeling culture protocol allowed us thus to efficiently uniformly ^{15}N-label and ^{13}C/^{15}N-label Abl using BV-infected insect cells. This labeling culture protocol combined with the herein employed labeling media should be applicable also to the isotope labeling of other rec. proteins as expression of other proteins in unlabeled BE2000-U medium was found to be sufficiently high. The method presented in this communication could therefore extend the range of recombinant proteins suitable for NMR studies also to those cases where successful expression is restricted to the Baculovirus expression system.

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