Supporting Information

© Copyright Wiley-VCH Verlag GmbH & Co. KGaA, 69451 Weinheim, 2010

Nondenaturing Chemical Proteomics for Protein Complex Isolation and Identification


cbic_201000574_sm_miscellaneous_information.pdf
Supporting Information

<table>
<thead>
<tr>
<th>Supporting Information</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>General methods</td>
<td>S1</td>
</tr>
<tr>
<td>General procedure for cleavage</td>
<td>S1</td>
</tr>
<tr>
<td>Experimental section</td>
<td>S2</td>
</tr>
<tr>
<td>Reduction of Novo-azo-Biotin probe</td>
<td>S2</td>
</tr>
<tr>
<td>Proteomic procedures</td>
<td>S3</td>
</tr>
<tr>
<td>DNA relaxation assay</td>
<td>S8</td>
</tr>
</tbody>
</table>

**General methods** All reagents were purchased from commercial suppliers and used without further purifications. Reactions were analyzed by thin layer chromatography (TLC) using plates cut from glass sheets (silica gel 60F-254 from Merck). Visualization was achieved under a 254 or 365 nm UV light and by immersion in an ethanolic solution of cerium sulfate, followed by treatment with a heat gun. Organic solutions were concentrated by rotary evaporation at 25-60 °C at 15-30 torr. UV-Vis spectra and kinetic were recorded on Shimadzu UV-1800 spectrophotometer. High resolution mass spectra (HRMS) were obtained using a Agilent Q-TOF (time of flight) 6520 and mass spectra using a Agilent MSD 1200 SL (ESI/APCI) with a Agilent HPLC1200 SL. The semi-preparative HPLC system consisted of a Waters 600 pump, a 2487 detector (Waters), a 5 ml sample loop and a Sunfire C18 column (150 mm × 19 mm i.d., 5 μm, Waters).

**General procedure for cleavage.** A 10 mM azo solution was prepared in DMSO and diluted in phosphate buffer (100 mM, pH 7.4) to obtain 50 μM of azo compound solution. Then, 950 μL of this solution was added to 50 μL of freshly prepared solution of sodium dithionite at 121 mM. Color disappearance was followed by UV/VIS spectrophotometry at 467 nm.

**Experimental section**

**Alkyn-HAZA-Biotin (1)**

Starting with 2-amino-5-iodobenzoic acid, the linker 1 was synthesized as previously reported by our group in 12 steps (3% of overall yield).\(^1\)

---

Novobiocin-azide (Novo-N₃)

Starting of novobiocin, Novo-N₃ was synthesized using described procedure in 5 steps from novobiocin (20% of overall yield).²

Novo-HAZA-Biotine (2)

Compound 1 (20.0 mg, 0.025 mmol) was suspended in a mixture of tBuOH/water (1:1) (0.4 mL). The reaction mixture was stirred and a solution of NovoN₃ (15.0 mg, 0.053 mmol) in DMSO (50 µL), copper(II) sulfate pentahydrate (1.4 mg, 0.005 mmol) and sodium ascorbate (2.2 mg, 0.011 mmol) were successively added. Stirring was continued for 16 h at room temperature. Solvents were evaporated under reduce pressure and residue was purified by preparative HPLC to give compound 2 as an orange oil (10 mg, 38%). ESI-MS 1637.2 [M+H]+. HRMS [M+H]+ m/z calcd 1637.7080, found 1637.7054.

Reduction of Novo-HAZA-biotin probe 2

Supplementary Figure 1: UV data recorded at 467 nm for the reduction of the probe 2 with dithionite (6 mM).

Supplementary Figure 2: LC-MS data of 2 after reduction with dithionite.

Proteomic procedures.

Thermus thermophilus Gyrase B, expression and purification.

The Gyrase B gene was amplified from Thermus thermophilus HB8 genomic DNA using Deep Vent polymerase and inserted into the NdeI-BamHI cloning sites of pET15b (Novagen). Plasmid-transformed E. coli BL21 DE3 cells were grown in LB medium at 37°C and IPTG (1 mM) was added at OD$_{600nm}$ 1 for 4h30min at 30°C to induce expression of the N-terminal 6His-tagged GyrB. The culture was then centrifuged and the cell pellet was washed in cold Phosphate Buffered Saline (PBS). Following resuspension in 20 mM Hepes pH 8, 500 mM NaCl, 5% glycerol, 1 mM PMSF, 1mM β-mercaptoethanol, protein cocktail inhibitor (ROCHE). The lysate was sonicated and then centrifuged at 18,000 rpm at 4°C for 1h. The supernatant was collected and loaded on a Hi-trap Ni-NTA affinity column (Pharmacia) and fractions containing GyrB were collected in the same buffer with 250 mM imidazole. Protein eluate was dialysed overnight in 10 mM Tris-Cl pH 8, 80 mM NaCl, 5% Glycerol, 0.1 mM EDTA, 1 mM DTT, at
4°C in the presence of thrombin protease for the removal of the histidine tag. Cleaved GyrB was purified by exchange ion Hi-trap Q-sepharose chromatography (Pharmacia) followed by gel filtration (Superdex 200, Pharmacia). Recombinant purified GyrB protein was resuspended in 10 mM Tris-Cl pH8, 200 mM NaCl, 20% Glycerol, 0.1 mM EDTA, 1 mM DTT and stored in aliquots at -80°C.

**Determination of non-denaturing dithionite concentration**

Purified Gyrase B (5 µg) was added in 30 µL of cleavage solution (Na$_2$S$_2$O$_4$ in PBS) at 0, 1, 5, 10, 25 mM and incubated for 15 min at RT. The reaction mixture was treated with native gel loading buffer (0.004% bromophenol blue, 20% glycerol, and water) and analyzed by electrophoresis in Tris-Glycine 7% non-denaturing acrylamide gels (Figure S3).

Supplementary Figure 3: Coomassie stained non-denaturing acrylamide gel of recombinant GyrB from *Thermus thermophilus* (5 µg) incubated with sodium dithionite. The black arrow shows the band corresponding to monomeric GyrB.

Lane 1: 0 mM, lane 2: 1 mM, lane3: 5 mM, lane 4: 10 mM, lane 5: 25 mM.

**General procedure of capture and release of purified Gyrase B**

50 µL of magnetic beads (Pierce 10 mg/mL) were washed three times with reaction buffer (Hepes 20 mM, NaCl 150 mM, MgCl$_2$ 2 mM, Tween$_{20}$ 0.1 %) using a magnetic separator. Then a solution of 2 (2.5 µM in reaction buffer, stock solution: 10 mM in DMSO) was incubated with magnetic beads for one hour at RT. Functionalized beads were washed three times with reaction buffer, then purified GyrB (50 µg) in reaction buffer was added to the beads. After 1.5 h of binding at RT, excess proteins were removed and magnetic beads were washed three times with reaction buffer to remove any unspecifically bound protein (The analysis of each eluted fractions by electrophoresis gel did not show premature cleavage of target protein and in the third wash no more protein was released). The resulting magnetic beads were resuspended in 30 µL of cleavage solution (Na$_2$S$_2$O$_4$ in Hepes 20 mM, NaCl 150 mM, MgCl$_2$ 2 mM, Tween$_{20}$ 0.1 %) for indicated concentration and times. Elution was repeated a second time. Supernatants were collected and treated with 3X SDS sample buffer. Next, streptavidine beads were washed with Hepes 20 mM, NaCl 150 mM, MgCl$_2$ 2 mM, Tween$_{20}$ 0.1 % buffer and boiled with SDS sample buffer
for 5 min at 95°C. Samples were loaded on a 10 % SDS-PAGE gel and proteins were determined by coomassie gel staining.

Optimization of dithionite concentration

Following the general procedure of capture and release described above, functionalized magnetic beads were divided in seven aliquots and resuspended twice in 30 µL of different cleavage solution (Na2S2O4 in Hepes 20 mM, NaCl 150 mM, MgCl2 2 mM, Tween20 0,1 %) at 0, 1, 2, 3, 4, 5 and 6 mM respectively. Resulting supernatants were loaded on a 10 % SDS-PAGE, and then the gel was stained with Coomassie Blue.

[Image: Supplementary Figure 4: Optimization of dithionite concentration on the cleavage yield. The Coomassie stained SDS-PAGE gel of recombinant GyrB from Thermus thermophilus was incubated with sodium dithionite. Lane 1: 0 mM, lane 2: 1 mM, lane 3: 2 mM, lane 4: 3 mM, lane 5: 4 mM, lane 6: 5 mM, lane 7: 6 mM]

[Graph: Optimization of dithionite concentration]

Supplementary Figure 5: Optimization of dithionite concentration
**Optimization of elution times**

Following the general procedure of capture and release described above, functionalized magnetic beads were divided in five aliquots and resuspended twice in 30 µL of a 5 mM cleavage solution (Na₂S₂O₄ in Hepes 20 mM, NaCl 150 mM, MgCl₂ 2 mM, Tween₂₀ 0.1%) for 0, 1, 5, 10, 15 minutes respectively. Resulting supernatants were loaded on a 10 % SDS-PAGE, and then the gel was stained with Coomassie Blue.

**Supplementary Figure 6**: Optimization of elution time. Coomassie stained SDS-PAGE gel of recombinant GyrB from *Thermus thermophilus* was incubated with sodium dithionite (5 mM). Lane 1: 0 min, lane 2: 1 min, lane 3: 5 min, lane 4: 10 min, lane 5: 15 min

![Optimization of elution time](image)

**Optimization of elution time**

**Supplementary Figure 7**: Optimization of elution time

**General procedure of capture and release of gyrase from cell lysate**

50 µL of magnetic beads (Pierce 10 mg/mL) were washed three times with reaction buffer (Hepes 20 mM, NaCl 150 mM, MgCl₂ 2 mM, Tween₂₀ 0.1%) using magnetic separator. Then a solution of 2 (2.5 µM in reaction buffer, stock solution 10 mM in DMSO) was incubated with magnetic beads for one hour at RT. Functionalized beads were washed three times with reaction buffer, then *E.Coli* cell lysate (10 mg) was added. After 1.5 h of binding at RT, excess proteins were removed and magnetic beads were washed three times with reaction buffer to remove unspecifically bound proteins. The resulting magnetic beads
were resuspended in 30 µL of cleavage solution (Na₂S₂O₄ 5 mM in Hepes 20 mM, NaCl 150 mM, MgCl₂ 2 mM, Tween® 0.1 %) for 5 minutes (or in buffer alone) and repeated a second time. Supernatants were collected and treated with 3X SDS sample buffer. Streptavidine beads were then washed with Hepes 20 mM, NaCl 150 mM, MgCl₂ 2 mM, Tween® 0.1 % buffer and boiled with SDS sample buffer for 5 min at 95°C. Samples were loaded on a 10 % SDS-PAGE gel and proteins were determined by Coomassie gel staining.

**MS-based protein identifications.**

*In gel* digestion of gel slices was performed as previously described.³ The resulting peptide extracts were analyzed by nanoLC-MS/MS on a nanoACQUITY Ultra-Performance-LC (UPLC, Waters, Milford, UK) coupled to a SYNAPT HDMS hybrid quadrupole-time-of-flight mass spectrometer (Waters, Milford, UK) equipped with a Z-spray ion source and a lock mass system. Peptide mixtures were loaded on a Symmetry C18 trap precolumn (180 µm inner diameter × 20 mm, particle size 5 µm; Waters, Milford, UK) and peptides were separated on a ACQUITY UPLC® BEH130 C18 column, 75 µm x 200 mm, 1.7 µm particle size (Waters, Milford, UK).

The solvent system consisted of 0.1 % formic acid in water (solvent A) and 0.1 % formic acid in acetonitrile (solvent B). Trapping was performed during 3 min at 5 µL/min with 99 % of solvent A and 1 % of solvent B. Elution was performed at a flow rate of 400 nL/min, using 1-50 % gradient (solvent B) over 35 min at 45°C followed by 65 % (solvent B) over 5 min.

The general mass spectrometric parameters were as follows: the capillary voltage was set at 3,500V and the cone voltage at 35V. Mass calibration of the TOF was achieved using [Glu1]-fibrinopeptide B (GFP) on the m/z 50-2000 range in the positive ion mode. Online correction of this calibration was achieved using lock-mass on product ions derived from the [Glu1]-fibrinopeptide B (GFP). The ion (M+2H)²⁺ at m/z 785.8426 is used to calibrate MS data and the fragment ion (M+H)⁺ at m/z 684.3469 is used to calibrate MS/MS data during the analysis. For tandem MS experiments, the system was operated with automatic switching between MS and MS/MS modes. The 4 most abundant peptides, preferably doubly and triply charged ions, were selected on each MS spectrum for further isolation and CID fragmentation. MS/MS fragmentation was performed using argon as collision gas. The complete system was fully controlled by MassLynx 4.1 (SCN 566, Waters, Milford, UK). Raw data collected during nanoLC-MS/MS analyses were processed and converted with ProteinLynx Browser 2.3 (23, Waters, Milford, UK) into *.pkl peak list format. The MS/MS data were analyzed using the MASCOT 2.2.0. algorithm (Matrix Science, London, UK) to search against the Swissprot database (57.13). Spectra were searched with a mass tolerance of 0.3 Da for MS and 0.3 Da for MS/MS data,

allowing a maximum of one missed cleavage with trypsin and with carbamidomethylation of cysteines and oxidation of methionines specified as variable modifications.

### DNA relaxation assay.

200 ng of pUC19 plasmid was incubated with 200 ng of DNA gyrase A2B2 in a buffer containing 20 mM Tris acetate pH8, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT and 1 mg/mL BSA to a final volume to 10 µL. The mixture was incubated 30 minutes at 37°C then the reaction was stopped with loading buffer containing 0.1% SDS. Reactions were loaded on a 1% agarose gel in TBE1X (Tris Borate EDTA) electrophoresed during 1.5 h at 120V. The gel was stained with ethidium bromide (0.5 µg/mL) and scanned with a Typhoon™ 8600 imager.

<table>
<thead>
<tr>
<th>Peptide start</th>
<th>Peptide sequence</th>
<th>Peptide Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>EITPVNIEEELK</td>
<td>1413.75</td>
</tr>
<tr>
<td>19</td>
<td>SSYDLYAMSVIGVR</td>
<td>1560.77</td>
</tr>
<tr>
<td>48</td>
<td>VLYAMVNLONWDNK</td>
<td>1636.82</td>
</tr>
<tr>
<td>69</td>
<td>VVGVDVIGK</td>
<td>786.472</td>
</tr>
<tr>
<td>77</td>
<td>YHPHDGSAYVDTIVR</td>
<td>1729.83</td>
</tr>
<tr>
<td>92</td>
<td>MAQPFSLR</td>
<td>949.43</td>
</tr>
<tr>
<td>100</td>
<td>YMVLDDGQNGFSIDGDSAAMR</td>
<td>2275.01</td>
</tr>
<tr>
<td>130</td>
<td>IAHELMADLEK</td>
<td>1269.65</td>
</tr>
<tr>
<td>141</td>
<td>ETVDVFVDNYDGTEK</td>
<td>1631.71</td>
</tr>
<tr>
<td>141</td>
<td>ETVDVFVDNYDGTEKPVDMPK</td>
<td>2513.18</td>
</tr>
<tr>
<td>155</td>
<td>IPDVMPK</td>
<td>900.487</td>
</tr>
<tr>
<td>228</td>
<td>GIEEAYR</td>
<td>837.4107</td>
</tr>
<tr>
<td>246</td>
<td>AEEVDAK</td>
<td>860.4366</td>
</tr>
<tr>
<td>257</td>
<td>ETIVHEIYPYGNK</td>
<td>1682.91</td>
</tr>
<tr>
<td>408</td>
<td>TALVANPWQLGNSAAMLER</td>
<td>2054.09</td>
</tr>
<tr>
<td>427</td>
<td>AGGDAOARPEWLEPEFGVR</td>
<td>2014.96</td>
</tr>
<tr>
<td>445</td>
<td>DGLYYLLEQGQAQADLR</td>
<td>2110.08</td>
</tr>
<tr>
<td>480</td>
<td>ELLODIÆELR</td>
<td>1312.75</td>
</tr>
<tr>
<td>504</td>
<td>EELELYR</td>
<td>887.4839</td>
</tr>
<tr>
<td>518</td>
<td>RITEITANSADILNEDLTIGEDVVTLSHQGYVK</td>
<td>3671.88</td>
</tr>
<tr>
<td>519</td>
<td>TEITANSADILNEDLTIGEDVVTLSHQGYVK</td>
<td>3515.78</td>
</tr>
<tr>
<td>551</td>
<td>YOPLSEYEAOQR</td>
<td>1383.65</td>
</tr>
<tr>
<td>572</td>
<td>IIEFEDFIR</td>
<td>1164.59</td>
</tr>
<tr>
<td>581</td>
<td>LLVANTHDILCFSSR</td>
<td>1882.96</td>
</tr>
<tr>
<td>604</td>
<td>VYQLPSEATR</td>
<td>1076.57</td>
</tr>
<tr>
<td>616</td>
<td>GRPVPILNPLEQDER</td>
<td>1748.97</td>
</tr>
<tr>
<td>631</td>
<td>ITAILPVTVEEGK</td>
<td>1645.91</td>
</tr>
<tr>
<td>646</td>
<td>VFMATANGTVK</td>
<td>154.59</td>
</tr>
<tr>
<td>657</td>
<td>KTLTEFNR</td>
<td>1107.62</td>
</tr>
<tr>
<td>658</td>
<td>TLVTETFNR</td>
<td>979.5213</td>
</tr>
<tr>
<td>713</td>
<td>AMGCNTTGGVR</td>
<td>1066.48</td>
</tr>
<tr>
<td>726</td>
<td>LGEKQGVKSLVPR</td>
<td>1481.87</td>
</tr>
<tr>
<td>732</td>
<td>VSVLSIVR</td>
<td>882.5777</td>
</tr>
<tr>
<td>755</td>
<td>RRTVEAEPYTK</td>
<td>1315.61</td>
</tr>
<tr>
<td>756</td>
<td>TAVAEYPTK</td>
<td>979.5101</td>
</tr>
<tr>
<td>780</td>
<td>NGLVGVGAVQVDDCDQIMMDTADGTLVR</td>
<td>2890.41</td>
</tr>
<tr>
<td>809</td>
<td>VSEISIGR</td>
<td>959.5526</td>
</tr>
<tr>
<td>818</td>
<td>NTOQGVLIR</td>
<td>1013.61</td>
</tr>
<tr>
<td>827</td>
<td>TAEDENVGQLR</td>
<td>1330.66</td>
</tr>
</tbody>
</table>

### DNA relaxation assay.

200 ng of pUC19 plasmid was incubated with 200 ng of DNA gyrase A2B2 in a buffer containing 20 mM Tris acetate pH8, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM DTT and 1 mg/mL BSA to a final volume to 10 µL. The mixture was incubated 30 minutes at 37°C then the reaction was stopped with loading buffer containing 0.1% SDS. Reactions were loaded on a 1% agarose gel in TBE1X (Tris Borate EDTA) electrophoresed during 1.5 h at 120V. The gel was stained with ethidium bromide (0.5 µg/mL) and scanned with a Typhoon™ 8600 imager.

<table>
<thead>
<tr>
<th>Peptide start</th>
<th>Peptide sequence</th>
<th>Peptide Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>SNSYDSSSSK</td>
<td>1087.49</td>
</tr>
<tr>
<td>77</td>
<td>GIPTGIHEEPEGVSAEVMVTLVHAGGK</td>
<td>2670.39</td>
</tr>
<tr>
<td>104</td>
<td>FDDNSYK</td>
<td>888.374</td>
</tr>
<tr>
<td>111</td>
<td>VSSGGLHGVGVSVNVNSQK</td>
<td>1808.00</td>
</tr>
<tr>
<td>130</td>
<td>LELVIOIR</td>
<td>870.541</td>
</tr>
<tr>
<td>143</td>
<td>QIYEHGVPQAPLAVGTEK</td>
<td>2167.10</td>
</tr>
<tr>
<td>193</td>
<td>ELSFLNSGVSR</td>
<td>1321.71</td>
</tr>
<tr>
<td>210</td>
<td>DGKEDHHFYYEGGK</td>
<td>1631.75</td>
</tr>
<tr>
<td>224</td>
<td>AVEVLNFK</td>
<td>983.502</td>
</tr>
<tr>
<td>232</td>
<td>NKSHPNYNFYSTEK</td>
<td>1936.00</td>
</tr>
<tr>
<td>234</td>
<td>TPHIPNHFYSTEK</td>
<td>1693.86</td>
</tr>
<tr>
<td>277</td>
<td>DGHTHALGFR</td>
<td>1030.51</td>
</tr>
<tr>
<td>292</td>
<td>TLAVMDK</td>
<td>955.456</td>
</tr>
<tr>
<td>317</td>
<td>EGIAVVSVK</td>
<td>1014.62</td>
</tr>
<tr>
<td>338</td>
<td>DKLVSSEVK</td>
<td>1004.56</td>
</tr>
<tr>
<td>347</td>
<td>SAVEQMNLELLAYLENPTDAK</td>
<td>2606.27</td>
</tr>
<tr>
<td>395</td>
<td>KGALDAIPLPGK</td>
<td>1139.68</td>
</tr>
<tr>
<td>396</td>
<td>KGALDAIPLPGK</td>
<td>1011.58</td>
</tr>
<tr>
<td>407</td>
<td>LACQERPDALSELYVEGDASGSGAK</td>
<td>2851.34</td>
</tr>
<tr>
<td>414</td>
<td>DPALSELYVEGDASGSGAK</td>
<td>1978.96</td>
</tr>
<tr>
<td>439</td>
<td>KGNAIPLPK</td>
<td>1024.65</td>
</tr>
<tr>
<td>440</td>
<td>NNQAIPLK</td>
<td>896.5571</td>
</tr>
<tr>
<td>461</td>
<td>MLSSOEVATLALGCGIGR</td>
<td>2077.08</td>
</tr>
<tr>
<td>481</td>
<td>TLLLTFFYR</td>
<td>1173.67</td>
</tr>
<tr>
<td>508</td>
<td>TPIHPNHFYSTEK</td>
<td>1693.86</td>
</tr>
<tr>
<td>517</td>
<td>OMIEWER</td>
<td>1017.50</td>
</tr>
<tr>
<td>525</td>
<td>GHHYIAQPYLPK</td>
<td>1385.76</td>
</tr>
<tr>
<td>535</td>
<td>LVSEYNATQK</td>
<td>1152.59</td>
</tr>
<tr>
<td>608</td>
<td>ELIYQIPITLLEADLSDEQTVRT</td>
<td>4222.20</td>
</tr>
<tr>
<td>629</td>
<td>WWNVLSELNDK</td>
<td>1387.72</td>
</tr>
<tr>
<td>629</td>
<td>WWNVLAVLSEDKEGHSQWK</td>
<td>2368.17</td>
</tr>
<tr>
<td>649</td>
<td>FDVTHAENQNLFEPIVR</td>
<td>2029.01</td>
</tr>
<tr>
<td>668</td>
<td>THGVDTIYDPLHDFETGGEYR</td>
<td>2422.10</td>
</tr>
<tr>
<td>699</td>
<td>GLLEEADFIER</td>
<td>1291.85</td>
</tr>
<tr>
<td>713</td>
<td>RQPASFEGALDWLVK</td>
<td>1887.01</td>
</tr>
<tr>
<td>714</td>
<td>QPVARSEFQALDWLVR</td>
<td>1730.91</td>
</tr>
<tr>
<td>741</td>
<td>GLGEMNPEOLWETTMDPESR</td>
<td>2320.02</td>
</tr>
<tr>
<td>769</td>
<td>DAIADOLFIITLMDGADEPR</td>
<td>2134.05</td>
</tr>
<tr>
<td>789</td>
<td>RAIEENALK</td>
<td>1190.65</td>
</tr>
<tr>
<td>790</td>
<td>AFIEENALK</td>
<td>1034.55</td>
</tr>
</tbody>
</table>