Recovery of Polypeptides Cleaved from Purified Calmodulin-Binding Peptide Fusion Proteins

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Purification of recombinant proteins has been greatly simplified in recent years because of the availability of expression vectors that allow fusion of the protein coding sequence of interest to short peptide sequences or larger proteins, enabling the affinity purification of the fusion protein from crude preparations. Fusion proteins are often engineered to contain short linkers of five to six amino acids, positioned between the affinity tag and the protein of interest, which serve as recognition targets for site-specific proteases thereby allowing the proteolytic removal of the affinity tag following purification of the fusion protein. The serine protease enterokinase (EK) is particularly attractive because it cleaves after the carboxy terminus of its recognition sequence $(Asp)_4$-Lys, allowing production of cleavage products that have native amino termini following the removal of N-terminal affinity tags. In addition, the recent cloning of bovine EK and its expression and purification from *E. coli* has allowed the production of high specific activity enzyme that is virtually free of contaminating proteases (1,5). At a low enzyme-to-substrate ratio (1:1000 wt/wt), fusion protein can often be cleaved to completion in a few minutes at 37°C, whereas with other site-specific proteases such as thrombin and factor Xa, cleavage reactions are usually carried out over a period of several hours.

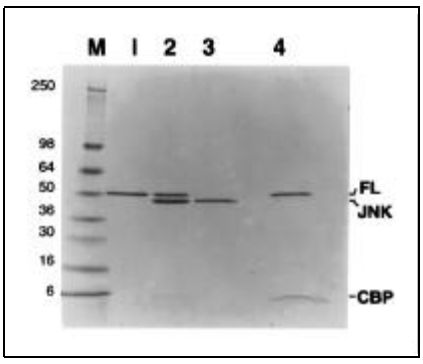

Figure 1. Removal of cleaved CBP affinity tag peptide fragment and uncleaved CBP fusion protein from EK digestion reactions. Purified CBP-EK-JNK (50 µg) (lane 1) was digested with 100 ng of EK in 100 µL of EK reaction buffer (50 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$ and 0.1% Tween® 20) for 3 min at 37°C and cooled on ice. Following removal of a portion of the reaction for SDS-PAGE analysis (lane 2), the sample was diluted to 500 µL with binding buffer (50 mM Tris-HCl, pH 8.0, 4 mM $CaCl_2$, 200 mM NaCl and 0.1% Tween 20) and added to a mixture of settled resin containing 50 µL CaM affinity resin and 20 µL STI-agarose (Sigma Chemical, St. Louis, MO, USA). The slurry was mechanically rotated at 4°C for 1 h. Following removal of unbound material (lane 3), a portion of the resin was boiled in Laemmli sample buffer (lane 4). Samples were boiled in Laemmli sample buffer and electrophoresed on a 4%–20% Tris-glycine SDS-PAGE (Novex, San Diego, CA, USA). The gel was stained with Coomassie® Brilliant Blue dye. M (molecular weight standards [kDa]), FL (full-length uncleaved CBP-EK-JNK), JNK (JNK polypeptide) and CBP (calmodulin binding peptide and EK recognition peptide fragment).

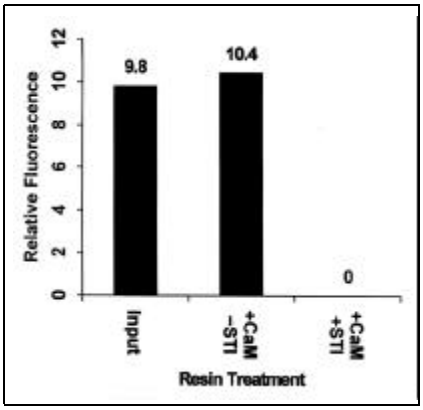

Figure 2. Determination of EK activity by fluorogenic peptide substrate assay. Purified CBP-EK-JNK (100 µg) was digested with 200 ng EK for 5 min at 37°C in a 1.0-mL reaction. The reaction was split into two 500-µL portions that were added to either 50 µL CaM-affinity resin (+CaM, -STI) or to a mixture of 50 µL CaM-affinity resin and 20 µL STI-agarose (+CaM, +STI). The resin slurries were mechanically rotated for 1 h at 4°C, and the resin was removed from the slurry by centrifugation. The unbound material was analyzed for EK activity using a fluorogenic peptide assay. The volumes of both fractions were adjusted to 420 µL with binding buffer, and the samples were mixed with 1.6 mL of a solution containing 0.5 mM of the fluorogenic EK substrate in 70 mM Tris-HCl, pH 8.0 and 10% dimethyl sulfoxide. Samples were mixed and immediately analyzed using a Model RF-1501 Spectrofluorometer (Shimadzu Scientific Instrument, Columbia, MD, USA). Enzyme activity was determined by measuring increased fluorescence (excitation 337 nm, emission 420 nm) due to the release of B-naphthylamine over a 1-min interval. For a positive control, 30 ng of EK were assayed (Input), and the increased fluorescence value was normalized to the amount of enzyme used in the experimental reactions.

Following purification of fusion proteins and proteolytic removal of the affinity peptide tags, it is often desirable to recover the mature cleavage product from EK reactions that contain a mixture of the protein of interest, the free affinity tag, EK and often small amounts of uncleaved fusion protein. Here we describe a simple and efficient one-step protocol for removing both EK and EK reaction products that contain the calmodulin-binding peptide (CBP) affinity tag (6,7).

The fusion protein CBP-EK-JNK was produced by fusing the five-amino acid EK target sequence to the N-terminus of the protein c-jun N-terminal kinase (JNK) (2,4,8) by polymerase chain reaction (PCR) and subsequently inserting the PCR fragment into the expression vector pCAL-n (6,8) so that the EK target peptide-JNK fusion protein was fused in-frame downstream from the CBP purification tag. The resulting fusion protein was expressed in BL21 (DE3) cells and purified from crude cell lysates to apparent homogeneity with calmodulin (CaM) affinity resin (Stratagene, La Jolla, CA, USA) (Figure 1, lane 1). Purified CBP-EK-JNK was partially digested by incubating with recombinant EK (Stratagene) at an enzyme-to-substrate ratio of 1:500 wt/wt for 3 min at 37°C. Using these conditions, slightly over half of the input fusion protein was cleaved to yield the 46-kDa JNK polypeptide and the 4 kDa CBP fragment (Figure 1, lane 2). Following the digestion, the reaction was adjusted to 4 mM $CaCl_2$ and 200 mM NaCl and mixed with 50 µL CaM-affinity resin and 20 µL soybean trypsin inhibitor-agarose (STI-agarose), the latter of which binds with high affinity to EK. The mixed resin slurry was mechanically rotated for 1 h at 4°C. Following absorption with the resin mixture, the unbound material was removed by centrifugation, and portions of the unbound supernatant and the resin pellet were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). As the results in Figure 1 indicate, the uncleaved CBP fusion protein and the cleaved CBP affinity tag were effectively removed by absorption with the resin mixture (Figure 1, compare lanes 2 and 3), while only a trace amount of cleaved JNK remained nonspecifically bound to the resin (lane 4). Protein concentration of the unbound material was assayed by the Coomassie® Plus Protein Assay (Pierce, Rockford, IL, USA), and the yield (25 µg) was determined to be approximately 83% of the JNK cleavage product present in the initial EK cleavage reaction, assuming that 60% of the input fusion protein was cleaved in the reaction.

The efficiency of EK removal with STI-agarose was determined by absorbing EK reaction mixtures, with either CaM-affinity resin alone or with a mixture of CaM-affinity resin plus STI-agarose, and assaying for EK activity

remaining in the unbound material using a fluorogenic peptide substrate assay (3). In this experiment, 200 ng of EK were used to digest CBP-EK-JNK at a 1:500 enzyme-to-substrate ratio in a 5-min reaction during which over 95% of the input fusion protein was cleaved as estimated by SDS-PAGE analysis (data not shown). After EK digestion of CBP-EK-JNK, the reaction was split and incubated with CaM affinity resin alone or with mixed resin as described above. To measure the amount of EK activity remaining in the supernatant, portions of the unbound material were mixed with a solution containing the fluorogenic peptide Gly-Asp-Asp-Asp-Asp-Lys-B-naphthylamide (Bachem Biosciences, Philadelphia, PA, USA). The samples were immediately analyzed in a spectrofluorometer to measure increased fluorescence due to release of B-naphthylamine over a 1-min interval. According to the results shown in Figure 2, there was no detectable EK activity remaining in the unbound fraction following incubation with STI-agarose (+STI, +CaM), while essentially all of the EK activity was recovered in the fraction that was absorbed with CaM-affinity resin alone (-STI, +CaM).

In this study, excess quantities of CaM-affinity resin and STI-agarose were used in the absorption experiments. The high affinity of both CBP fusion proteins and EK for their respective affinity resins using the mutually compatible binding conditions used in these experiments, together with the high binding capacities of the resins (both resins bind 1.5–3.0 mg of protein per mL of swollen resin) should allow this technique to be effectively used for large-scale purifications without requiring the use of large quantities of resin or excessive absorption times and should result in the rapid and efficient high yield recovery of purified EK cleavage products that have native amino acid sequence without any extraneous amino acid residues derived from the affinity tag or protease target site.

REFERENCES

1. **Collins-Racie, L.A., J.M. McColgan, K.L. Grant, E.A. DiBlasio-Smith, J.M. McCoy and E.R. LaVallie.** 1995. Production of recombinant bovine enterokinase catalytic subunit in *Escherichia coli* using the novel secretory fusion partner DsbA. Bio/Technology *13*:982-987.
2. **Derijard, B., M. Hibi, I.-H. Wu, T. Barrett, B. Su, T. Deng, M. Karin and R.J. Davis.** 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell *76*:1025-1037.
3. **Grant, D.A.W. and J. Hermon-Taylor.** 1979. Hydrolysis of artificial substrates by enterokinase and trypsin and the development of a sensitive specific assay for enterokinase in serum. Biochim. Biophys. Acta *567*:207-215.
4. **Kyriakis, J.M., P. Banerjee, E. Nikolakaki, T. Dai, E.A. Rubie, M.F. Ahmad, J.U. Avruch and J.R. Woodgett.** 1994. The stress-activated protein kinase subfamily of c-jun kinases. Nature *369*:156-158.
5. **LaVallie, E.R., A. Rehemtulla, L.A. Racie, E.A. DiBlasio, C. Ferenz, K.L. Grant, A. Light and J.M. McCoy.** 1993. Cloning and functional expression of a cDNA encoding the catalytic subunit of bovine enterokinase. J. Biol. Chem. *268*:23311-23317.
6. **Simcox, T.G., C.-F. Zheng, M.E. Simcox and P. Vaillancourt.** 1995. Calmodulin binding peptide affinity tag *E. coli* protein purification system. Strateg. Mol. Cell Biol. *8*:40-43.
7. **Stofko-Hahn, R.E., D.W. Carr and J.D. Scott.** 1992. A single step purification for recombinant proteins. FEBS Lett. *302*:274-278.
8. **Zheng, C.-F. and T.G. Simcox.** 1996. Recombinant c-jun N-terminal kinase (JNK) for studying cellular stress signal transduction in mammalian cells. Strateg. Mol. Biol. *9*:20-21.

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