High Speed Separation of Isozymes of Recombinant cAMP-Dependent Protein Kinase Using UNO™ S1 and S6 Columns

An improved method for the purification of crude C_α-subunit containing cell lysate was developed with an UNO S1 column (Bio-Rad catalog # 720-0021). The effect of flow rate on the resolution was explored using an UNO S6 column (Bio-Rad catalog # 720-0023).

cAMP-dependent protein kinase (cAMP) is one of the simplest and best understood enzymes in the diverse family of protein kinases.[1] The inactive holoenzyme contains two regulatory (R), and two catalytic (C), subunits. The complex is dissociated in response to nanomolar concentrations of the second messenger, cAMP, yielding an $R_2(cAMP)_4$ dimer and two active monomeric C-subunits.

The C_α-subunit can be expressed readily in *E. coli*[2] and this recombinant enzyme, rC_α, is very similar to the mammalian C_α-subunit in its enzymatic properties. The recombinant catalytic subunit (rC_α) of cAMP-dependent protein kinase was separated into three distinct isoforms using UNO S1 and S6 cation exchange columns.

The rC_α-subunit copurifies on P11 chromatography with a bacterial protein (called L11) and subsequent high resolution cation exchange chromatography using UNO S columns is required to remove this protein and separate the isozymes into distinct phosphoforms.

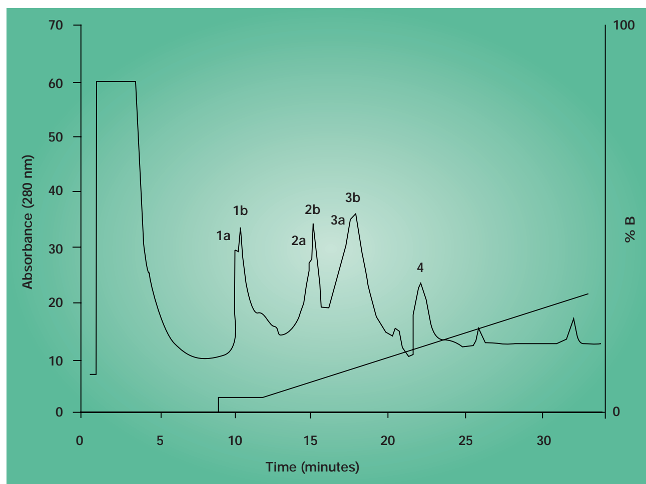

Fig. 1. *Sample:* E. coli *lysate; Column: UNO S1; Buffer A: 20 mM potassium phosphate, 1 mM DTT, pH 6.6; Buffer B: A+1 M KCl, pH 6.6; Gradient: 0% B in 17.5 ml, 5% B in 7.5 ml, 5–22% in 40 ml 22–100% B in 20 ml; Flow rate: load 1 ml/min, elute 2 ml/min; Detection, 280 nm.*

Improved Purification of Crude Lysate on UNO S1

Wild-type recombinant C-subunit was expressed in *E. coli* BL21.DE3 cells using a pT7-7 vector containing cDNA for the mouse C_α-subunit. Cells were lysed in a French press and the supernatant was buffer exchanged into 20 mM potassium phosphate, 1 mM DTT, pH 6.6, and was applied to the UNO S1 column at 1 ml/min and eluted at 2 ml/min. Protein was eluted using a 1 M KCl gradient. Fractions were analyzed using SDS-PAGE, IEF, and by electrospray mass spectroscopy. Kinase activity from the UNO S1 column was measured in 10 mM $MgCl_2$, 1 mM ATP and 100 mM MOPS, pH 7.0. Purified LRRASLG was used as the substrate.

Results

When the crude lysate was fractionated on the UNO S1 column, four major peaks were obtained in the gradient (Figure 1). SDS-PAGE of the peaks, lanes 10–14, shows that they contained bands 42 kDa in size (Figure 2). Isoelectric focusing of the fractions confirmed that major peaks 2, 3, and 4 corresponded to rC_α-subunit isozyme I, isozyme II, and isozyme III, respectively (Figure 3). Mass spectroscopy of the peaks indicated that the isozymes differed by a phosphate group and the values corresponded to those stated in the literature (Figure 4).[1] The specific activity for peaks 1b, 2, and 3 was 27, 21, and 45 U/mg, respectively which corresponds to the ~25 U/mg obtained for rC_α-subunit purified using the combination of P11 phosphocellulose and a conventional cation exchange column.[1]

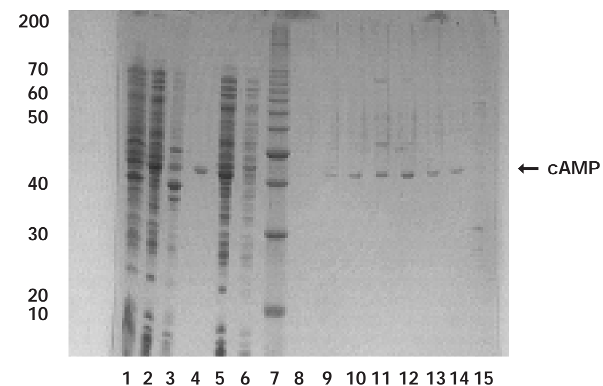

Fig. 2. *SDS-PAGE of UNO S1 fractions. Lane 1, Crude. Lane 2, Supernatant. Lane 3, Pellet. Lane 4, cAMP standard. Lane 5, Supernatant/filtered in buffer A. Lane 6, flowthrough. Lane 7, marker 10 kDa Gibco. Lane 8, wash. Lane 9, wash. Lane 10, Iso 1. Lane 11, Iso 1. Lane 12, Iso 2. Lane 13, Iso 2. Lane 14, Iso 3. Lane 15, High salt strip.*

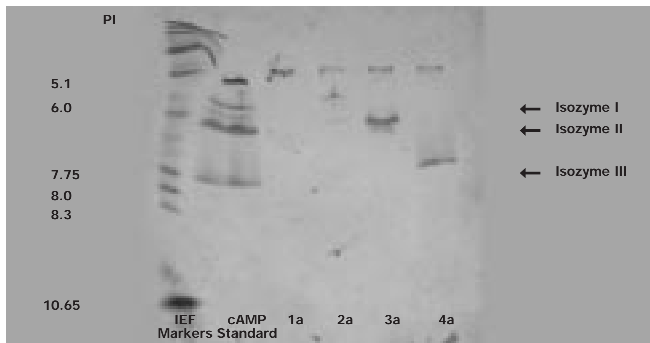

Fig. 3. *Isoelectric focusing gel of the fractions from UNO S1 separation.*

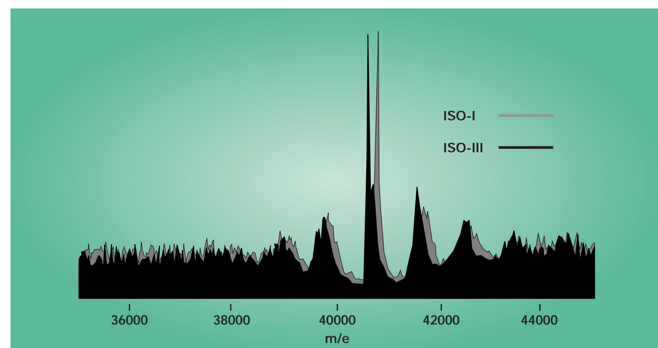

Fig. 4. *Electrospray mass spectra of three cAMP-dependent protein kinase isoforms from UNO S1 separation. Isozyme 1=40,762 daltons, Isozyme II - 40,683 daltons, Isozyme III=40,600 daltons. Data for Isozyme II not shown.*

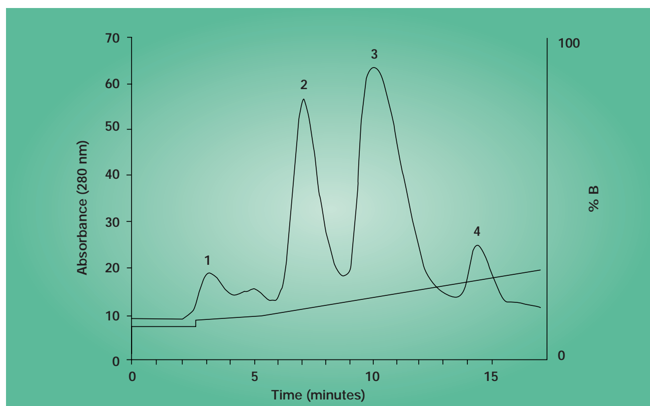

Fig. 5. *Purification of P11 phosphocellulose fraction on UNO S6 at 2 ml/min. Buffer A: 20 mM potassium phosphate, 1 mM DTT, pH 6.6; Buffer B: A+1 M KCl, pH 6.6; Gradient: 8–22% in 30 ml; Detection, 280 nm.*

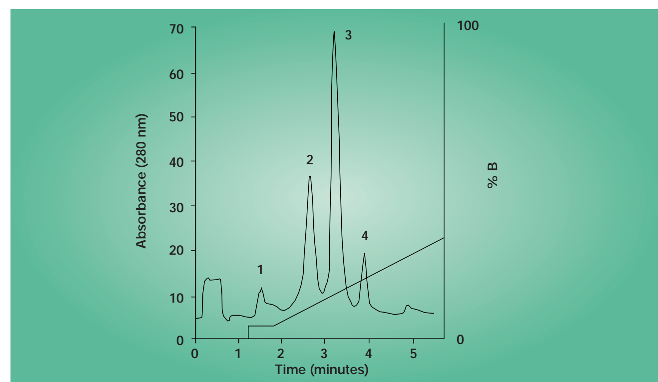

Fig. 6. *Purification of P11 phosphocellulose fraction; on UNO S6 at 10 ml/min. Buffer A: 20 mM potassium phosphate, 1 mM DTT, pH, 6.6; Buffer B: A+1 M KCl pH, 6.6; Gradient: 7–20% in 25 ml; Detection, 280 nm.*

Table I. Summary of rC_α-Subunit Fractionation on UNO S1 and S6 columns

COLUMN	FLOW RATE (ml/min)	SAMPLE VOLUME (ml)	SAMPLE LOAD (mg)	RECOVERED ISOFORMS (mg)	% RECOVERY	RESOLUTION PEAK I, II	RESOLUTION PEAK II, III
UNO S1	2	2	-	-	-	-	-
UNO S6	2	30	19.2	7.9	41	1.1	1.6
UNO S6	10	10	6.4	2.6	41	1.5	1.9

Flow Rate Effect on UNO S6

The *E. coli* lysate was diluted and fractionated on a P11 phosphocellulose support. The rC_α-subunit-containing fraction was dialyzed into 20 mM potassium phosphate, 1 mM DTT, pH 6.6, and was applied to the UNO S6 column. The sample was fractionated at 2 and 10 ml/min using a 1 M KCl gradient. Fractions were analyzed using SDS-PAGE.

To explore the effects of changing the flow rate, the P11-purified rC_α-subunit fraction was separated on the UNO S6 column at 2 ml/min and at 10 ml/min as shown in Figures 5 and 6. There is an increase in resolution when the flow is increased to 10 ml/min.

The enhanced resolution at the higher flow rate may be a result of the smaller sample loading and increased mass transfer effects (Table I). Protein recovery was 41%.

References

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Data courtesy of Dr. Friedrich Herberg and Marco De Stefano, University Bochum, Germany.

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