Purification of a Calcium-Binding Protein using CHT™ Ceramic Hydroxyapatite

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Introduction

Frequenin is a 22 kD calcium-binding protein found in neurons of vertebrates and invertebrates (Pongs et al. 1993, Olafsson et al. 1995, 1997). Frequenin appears to be particularly enriched in synapses, such as the motor nerve endings at neuromuscular junctions, and studies in vertebrates suggest a role in neurotransmission at neuromuscular synapses (Pongs et al. 1993, Olafsson et al. 1995). Like other calcium-binding proteins, it has 4 potential calcium-binding domains, or EF-hands (Olafsson et al. 1995). The N-terminus contains a consensus sequence for myristoylation, which is important for calcium-dependent membrane targeting (McFerran et al. 1998). Expression of frequenin is altered under a variety of pathological conditions like traumatic injury and axotomy (Jeromin et al. in preparation). Thus, understanding the structure-function properties of this and related proteins is of much interest.

Xenopus and rat frequenin have been cloned as hexahistidine proteins and purified by immobilized metal affinity chromatography (Olafsson et al. 1995, McFerran et al. 1998), but purification of the native species has not been reported. Because myristoylation will not occur with hexahistidine at the N-terminus (unpublished data), an alternate purification procedure was required to provide native protein for biophysical and physiological studies. We reasoned that frequenin may have a high affinity for the calcium sites in CHT ceramic hydroxyapatite, and that it would thus exhibit longer retention times than most potential contaminating species. This report describes the purification of frequenin in a single step on CHT.

Results

Frequenin was overexpressed in *Escherichia coli*. The lysate was dialyzed overnight against 10 mM sodium phosphate, pH 6.8 (Buffer A), and clarified by 0.45 µm filtration. The filtrate was applied directly to a 5 ml Bio-Scale CHT-I column (particle size 10 µm, Bio-Rad catalog # 751-0023) equilibrated with Buffer A. The column was washed with Buffer A until the unbound fraction eluted and then a linear gradient up to 400 mM sodium phosphate, pH 6.8, was initiated. The gradient eluted a complex cluster of peaks followed by a single, well-resolved species (Figure 1).

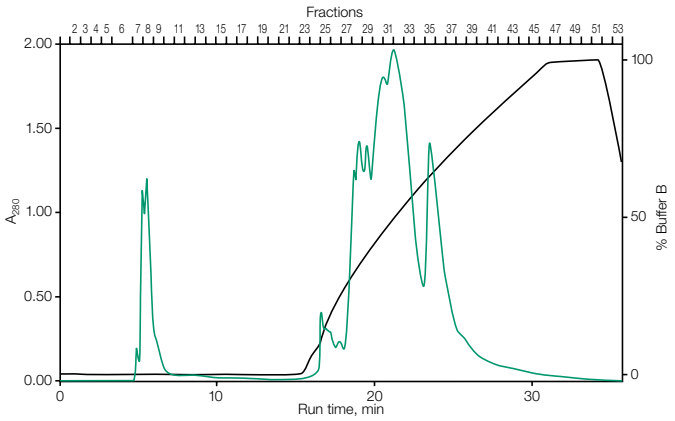

Fig. 1. Purification of frequenin on CHT-I. Column, 10 x 64 mm; load, 0.5 ml lysate. Buffer A, 10 mM sodium phosphate, pH 6.8; Buffer B, 400 mM sodium phosphate, pH 6.8; gradient, 0–100% Buffer B in 10 column volumes; flow rate, 2.0 ml/min; fraction size, 2.0 ml. Green trace, A_{280}; black trace, actual % Buffer B.

SDS-PAGE analysis of the CHT-I column fractions showed that the expression level of frequenin was relatively high (Figure 2, lanes 2 and 9). This observation and the molecular weight (MW) marker proteins allowed us to identify the last peak as the frequenin, which was highly purified (Figure 2).

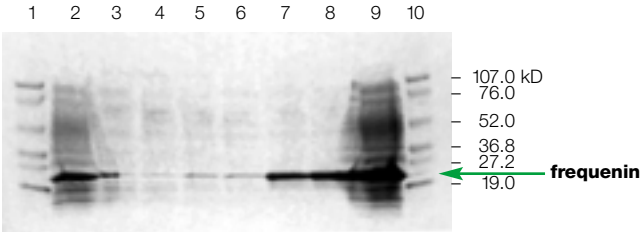

Fig. 2. SDS-PAGE of frequenin fractions from Figure 1. Gel, 4–20% acrylamide; lanes 1 and 10, Bio-Rad low-range MW standards; lanes 2 and 9, load; lanes 3–8: fractions 31–36. Gel stained with Coomassie Blue.

Myristoylated frequenin was purified by an identical protocol with very similar results. The identity and integrity of the purified proteins were confirmed by mass spectroscopy and western blotting (data not shown). The procedures were subsequently scaled up some 4-fold and then 50-fold. Again, very similar results were obtained. A 20 ml CHT-I

column yielded 22.1 mg of frequenin and 6.42 mg of myristoylated frequenin from 5.0 and 7.0 ml of *E. coli* lysate, respectively, assuming an A_{280} of 0.948 for a 0.1% solution. Figure 3 shows the chromatogram obtained during frequenin purification from 26 ml of *E. coli* lysate on a 100 ml CHT-I column packed with bulk medium (particle size 20 µm, catalog # 157-0020). Purity assessed by SDS-PAGE was similar to that obtained at small scale although some sample degradation was apparent (Figure 4). Some tailing of the frequenin into earlier fractions was also apparent, possibly due to the high nucleic acid content of the lysate.

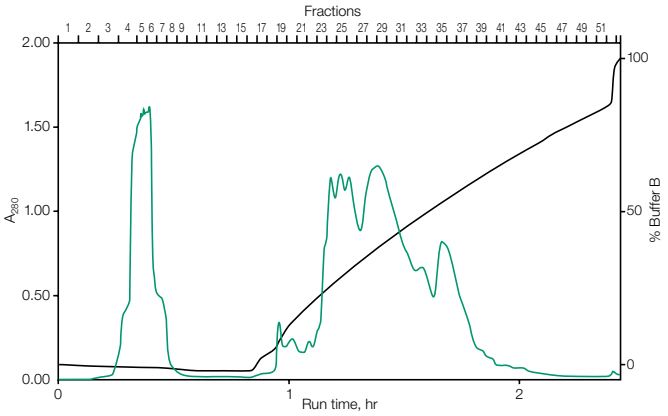

Fig. 3. Scale-up of frequenin purification on CHT-I. Column, 3.2 x 12.5 cm; load, 26 ml lysate diluted to 100 ml. Buffer A, 10 mM sodium phosphate, pH 7.0; Buffer B, 500 mM sodium phosphate, pH 7.0; gradient, 0–100% B in 10 column volumes. Flow rate was 5.0 ml/min during load, 10.0 ml/min during elution; collected fraction size, 26 ml. Green trace, A_{280}; black trace, actual % Buffer B.

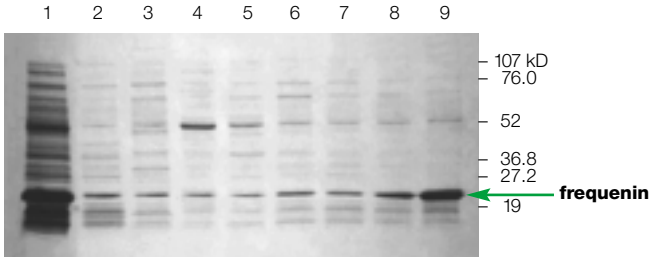

Fig. 4. SDS-PAGE of frequenin fractions from Figure 3. Gel, 4–20% acrylamide; lane 1, load; lanes 2–9, fractions 5, 20, 23, 25, 27, 29, 31, 36. Gel stained with Coomassie Blue.

Discussion

The results reported here indicate that CHT chromatography can purify a neuronal calcium-binding protein essentially to homogeneity in a single step. Calretinin, calbindin and a calcium-dependent adhesin from *Rhizobiaceae* are other calcium-binding proteins that have been purified using CHT (Cheung et al. 1993, Smit et al. 1989), while osteonectin has been reported to bind to hydroxyapatite (Kopp et al 1992). The process was shown to be easily scaled up from a 2.0 ml, 10 µm-particle analytical column to a 100 ml, 20 µm-particle preparative column with nearly identical results. More than 250 proteins are grouped in the EF-hand calcium-binding superfamily alone (Braunewell and Gundelfinger 1999), which adds to the potential utility of CHT for the rapid, facile isolation of physiologically important protein species at the analytical and preparative levels (Kopp et al. 1992).

References

Braunewell K-H and Gundelfinger ED, Intracellular neuronal calcium sensor proteins: a family of EF-hand calcium-binding proteins in search of a function, Cell Tissue Res, 295, 1–12 (1999)

Cheung WT et al., Calcium binding by chick calretinin and rat calbindin D28k synthesized in bacteria, Eur J Biochem, 215, 401–410 (1993)

Kopp JB et al., Renal tubular epithelial cells express osteonectin in vivo and in vitro, Kidney Int, 41, 56–64 (1992)

McFerran BW et al., Neuronal Ca^{2+} sensor 1, the mammalian homologue of frequenin, is expressed in chromaffin and PC12 cells and regulates neurosecretion from dense-core granules, J Biol Chem, 273, 22768–22772 (1998)

Olafsson P et al., Molecular cloning and functional characterization of the *Xenopus* Ca^{2+}-binding protein frequenin, Proc Natl Acad Sci USA, 92, 8001–8005 (1995)

Olafsson P et al., The Ca^{2+} binding protein, frequenin is a nervous system-specific protein in mouse preferentially localized in neurites, Brain Res Mol Brain Res, 44, 73–82 (1997)

Pongs O et al., Frequenin – a novel calcium-binding protein that modulates synaptic efficacy in the *Drosophila* nervous system, Neuron, 11, 15–28 (1993)

Smit G et al., Purification and partial characterization of the *Rhizobium leguminosarum* biovar *viciae* Ca^{2+}-dependent adhesin, which mediates the first step in attachment of cells of the family *Rhizobiaceae* to plant root hair tips, J Bacteriol, 17, 4054–4062 (1989)

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