EXPRESSION, PURIFICATION AND CHARACTERIZATION OF A HUMAN CALCIUM-BINDING PROTEIN: HUMAN CENTRIN 2

By

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Abstract

Human centrin 2 (Hcen 2) is an acidic centrosomal protein of 19,738 Da, that is comprised of four subdomains, called EF-hands, which represent the calcium-binding sites. Human centrin 2 gene locus is codified on chromosomes X and had been designated as *Hcetn2*. Hcen2 may play a role in centrosome duplication and segregation during mitosis. Transformed bacterial *E. coli* cells with a recombinant molecule containing the Hcetn2 gene were grown in either 2xYT broth or ^{13}C isotope enriched minimal media and induced to over-express the Hcen 2 and ^{13}C-Hcen 2, respectively. These recombinant proteins were then purified by using hydrophobic affinity chromatography followed by several anion exchange chromatographic separations. Highly pure Human centrin 2 and ^{13}C-homogenously labeled protein were obtained for biophysical studies. Differential scanning calorimetric (DSC) study of Hcen 2 showed that this is a very stable protein with a pre-transition temperature around 85 ^{o}C and a thermal transition temperature of 120 ^{o}C.

Resumen

La centrina humana 2 (Hcen 2) es una proteína acídica del centrosoma con un peso molecular de 19, 738 Da. Está compuesta por cuatro sub-dominios llamados manos de EF, los cuales representan sitios de enlace para calcio. El gen de la centrina humana 2 está codificado en el cromosoma X y ha sido nombrado como *Hcetn2*. Hcen 2 parece desempeñar un papel importante en la duplicación y segregación del centrosoma durante la mitosis. Células bacterianas competentes *E. coli* fueron transformadas con el gen que codifica para *Hcetn2* y crecidas en dos tipos de caldos de cultivo: 2xYT y en un medio mínimo enriquecido marcado con ^{13}C. Luego se indujeron para sobre-expresar las proteínas Hcen 2 y ^{13}C-Hcen 2. Dichas proteínas fueron purificadas usando cromatografía de afinidad hidrofóbica, la cual fue seguida por varios pasos de separación mediante cromatografía de intercambio aniónico. La centrina humana 2 y su forma marcada con ^{13}C fueron obtenidas con gran pureza para realizar estudios biofísicos. Los estudios de calorimetría de rastreo diferencial de la centrina humana 2 muestran que esta es una proteína estable con una pre-transición a 85 ^{o}C y una temperatura de desnaturalización de 120 ^{o}C.

To my Family…thanks for encouraging me to want more and do more,

for supporting me in the worst

and most importantly for all your love.

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Acknowledgement to the Mayo clinic and Foundation DNA core facility, the Protein core facility and to Vanderbilt University Mass Spectral Facility for their services in sequencing and mass spectral analysis of Hcetn2 and ^{13}C-Hcen2, respectively.

My gratitude and my heart to my “teachers” and coworkers in the day by day work at the “lab”: Zuleika, Danny, Sindy, Jessica, Lili, Muriel (you know for how much…), Christina and Arnaldo.

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1 Introduction

The cell division has been an interesting issue along the history of biology and the participation of the centrosome in this process has been recognized since its first description in 1876 by Van Beneden. Until now there is enough evidence that the centrosome and its pair of centrioles organize the number and polarity of cytoplasm microtubules. Once in each cell cycle, the centrosome is duplicated to give rise to two centrosomes that organize the microtubule array of the mitotic spindle, which makes possible equal segregation of sister chromatids into each of the two daughter cells. Some of the remaining questions in cell biology are: How cells generate only one new centrosome in each cell cycle? Why is cell division disorganized in cancerous cells?

In order to answer these questions we have to understand the regulation of expression of the centrosome precursor proteins and their post-translational modification and how these proteins interact with each other to conduct a normal process of chromosome segregation between daughter cells. This research proposes to study Human centrin 2, an interesting protein which is not only express, but also actively functional in all human somatic cells as a component of the centriolar and pericentriolar material; and that has shown to be required for the normal duplication and separation of the centrioles in human cultured cells (1).

Recently, aberrant centrin phosphorylation has been shown to occur in human breast tumor (2); which is the most common cause of cancer in American women. The number of new cases of breast cancer in American women was estimated to be about

212,600 in 2003 (3). It is extremely important to express and characterize this protein in order to fully understand the organization of cell division in normal cells and the cause of the disorganization observed in solid tumor cells, such as breast cancer cell.

In the past our laboratory has focused in the study of *Chlamydomonas* centrin which share an 80% identity with human centrin 1 and 2 and has contributed to the knowledge of its secondary structure in the presence and absence of cations (4) and to the understanding of the protein accessibility to its aqueous environment (5). Here, we turned our interest into the study of a human isoform, dealing with the optimization of its induction, over-expression and the purification processes based on the previously reported biochemical characteristic of the protein. We were also interested on dealing with partial characterization of the protein.

Objectives

1. Achieve a positive transformation of bacterial *E. coli* BL21-Gold (DE3) codon plus competent cells with a recombinant molecule encoding Human centrin 2 wild type protein.

2. Induce and over express wild type Human centrin 2 in bacterial cells by the use of a bioreactor, which allow us to closely monitor and control the environmental condition to ensure the best quality results and a great yield of protein expression.

3. Obtain milligrams quantities of highly pure Human centrin 2 and ^{13}C isotope homogenously labeled Human centrin 2 for biophysical studies through a simple and reliable purification process.

4. Analyze the calorimetric properties of the centriole associated protein Human centrin 2.

2 Literature Review

The number, direction and polarity of microtubules in eukaryotic cells are organized by a unique microtubule organizing center (MTOC). The MTOC of mammalian cells is the centrosome, which consists of a pair of centrioles surrounded by pericentriolar material that connects the centrioles to one another and to microtubules (1).

On three occasions during the cell cycle centrioles move apart from one another. The first separation occurs later in G_1 phase, as cells pass the restriction point and the pair of centrioles become disoriented and slightly separated from one another in the first identifiable event of centrosome duplication. The second, separation occurs as cells enter prophase and newly duplicated centrosomes (each containing a pair of centrioles) separate and migrate to opposite sites of the nucleous where they function as mitotic spindle poles. The third event occurs immediately following mitosis, when the centrioles pair transiently splits during or just after telophase. At this time, the older centriole remains near the cell center, while the younger centriole wanders extensively throughout the cytoplasm before returning to reside near its older partner. Changes in centrosome function at the time of G_2/M transition are regulated by phosphorylation; and increase in protein phosphorylation has been observed at this time (6).

There has been some progress in understanding centrosome function and composition, but at the present time, only a few of the estimated 150-200 centrosome and mitotic spindle pole proteins are known and are well characterized. These include: centrin, which functions in centrosome duplication and separation; γ-tubulin, a unique

member of the tubulin family that plays a role in microtubule nucleation; and pericentrin, a protein involved in organizing centrosome structure. Centrin is located within the centrioles themselves and, along with γ-tubulin and pericentrin, is also a component of the pericentiolar (Hcen3) material that surrounds the centrioles (2).

Centrin, a calcium binding phosphorilated-protein, was first identified at the contractile flagellar roots of green alga. Centrin has since been found to be a ubiquitous protein associated with centrioles/basal bodies, centrosome and mitotic spindle poles in cells from diverse evolutionary lineages (7). Nonetheless, centrin is one of approximately 350 proteins that are unique to eukaryotic cells and have no significant homology to proteins in Archaea and bacteria (1).

Vertebrate centrin is 172 amino acids in length, and among vertebrates has 80-90% amino acid sequence identity; whereas centrin sequences of lower organisms show 50-70% amino acid identity when compared to vertebrate centrin.

The amino acid sequence analysis reveals that centrin represents a highly conserved, yet distinct, subgroup of the EF-hand superfamily of calcium-binding proteins. The helix-loop-helix, EF-hand motif, is the canonical signature for cytoplasmic calcium binding proteins. Integral centrins are composed of two independent domains, each containing two EF-hand consensus motifs, and these regions of the protein are the most highly conserved feature of the molecule between different centrin species, and between centrin and other members of the EF-hand superfamily. In *Chlamydomonas*

centrin, all four EF-hands bind Ca^{2+} at physiological levels, whereas Human centrin 2 exhibits one strong (loop IV) and one weak binding site (loop III), localized in the C-terminal domain (8, 9) (Figure 2.1).

The amino-terminal subdomain is the most distinctive and variable region in the centrin sequence as shown in Figure 2.1. It has been suggested that this domain confers centrin functional diversity. The amino terminal 15-24 amino acids is the most distinctive and variable feature among centrin from various species. The carboxy terminal end of the protein has the conserved phosphorylation consensus sequence KKTS^{p}Y for protein kinase A. The EF-hands of centrin are in exact sequence register with those found in calmodulin. Due to their high homology and identical domain architecture, the tertiary structure of centrin may be similar to that of calmodulin (4, 7, 10) Figure 2.2.

In humans, there are at least three centrin isoforms with variable sequences: Human centrin 1 (Hcen 1) coded on chromosome 14 (11), Human centrin 2 (Hcen 2) coded on chromosome X (12) and Human centrin 3 (Hcen 3) coded on chromosome 5 (13). Hcen 1 is exclusively expressed in male germ cells, while Hcen 2 and Hcen 3 are expressed in somatic cells. Centrin 2 is a centriole protein and recombinant GFP- centrin 2 localizes to centrioles throughout the cell cycle, while Hcen 3 localizes to the pericentriolar material (1). Hcen 1 and Hcen 2 are highly similar to each other (sequence identity 84%) and to the algae centrin (68 and 71%, respectively), whereas Hcen 3 has a more distant sequence; it shows only 54% identity with both Hcen 1 and Hcen 2, and is

slightly closer to Cdc31, the centrin equivalent in yeast *Saccharomyces cerevisiae* (59% sequence identity).

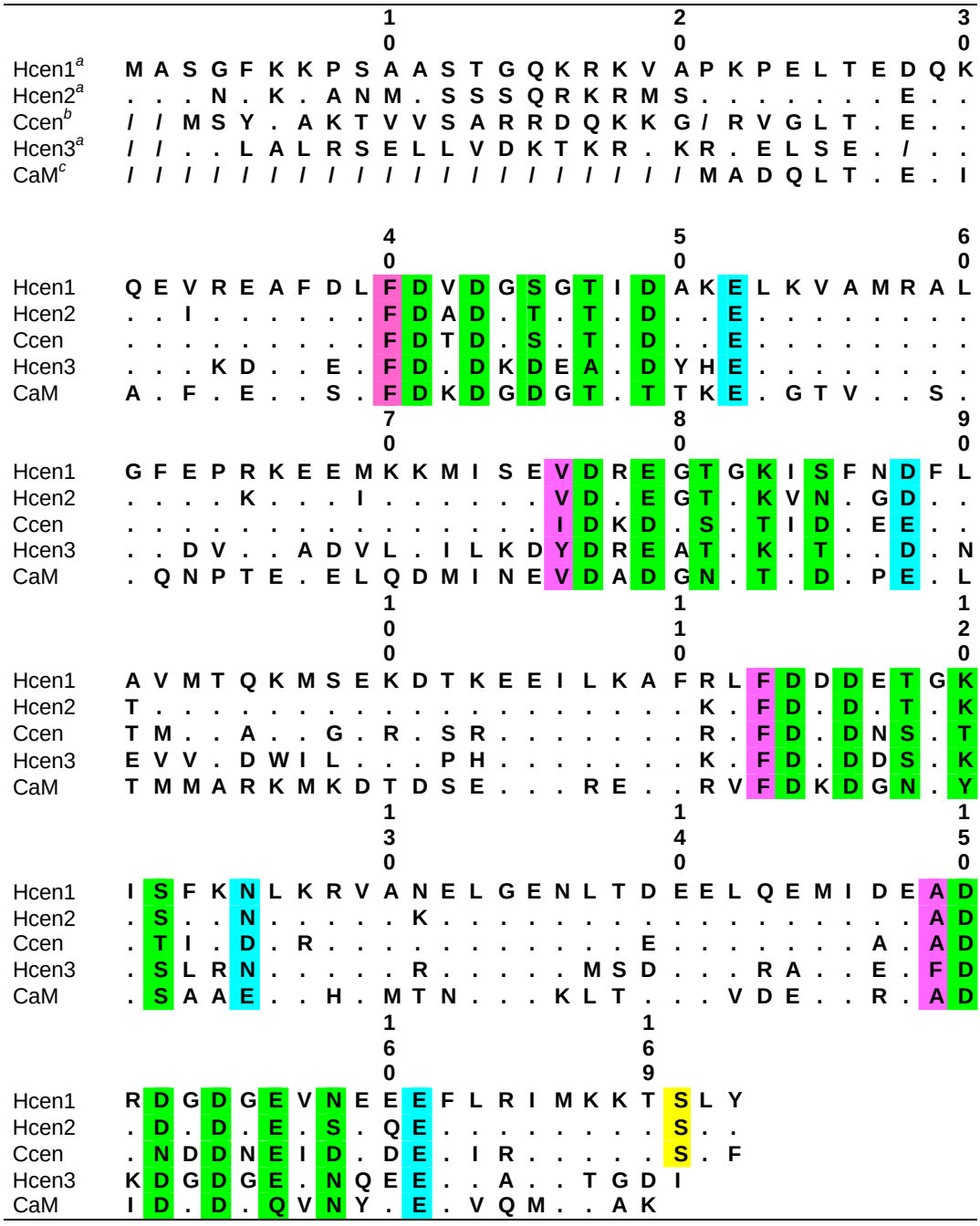
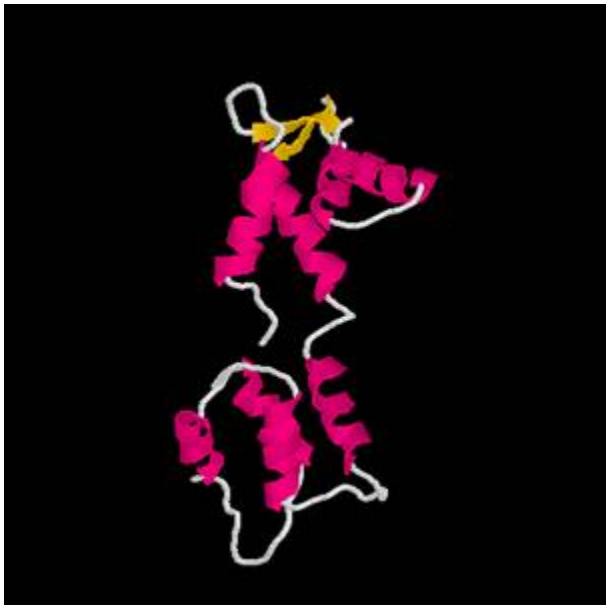


Figure 2.1: Amino acid sequence of Human centrin isoforms: this figure compares the amino acid sequence among [a] Human centrins 1, 2 and 3, [b]*Chlamydomonas* centrin, and [c]Calmodulin. When compared to previous sequence identical amino acids are denoted with a dot (.); introduced gaps in sequence are denoted with a slash (/).
Residues involved in calcium binding site are green and blue; hydrophobic residues at the beginning of calcium binding sequence are purple, and SER 170 (yellow) is the phosphorylation site. This Figure was provided by Dr. Pastrana.

apo-CaM



Ca_4: CaM

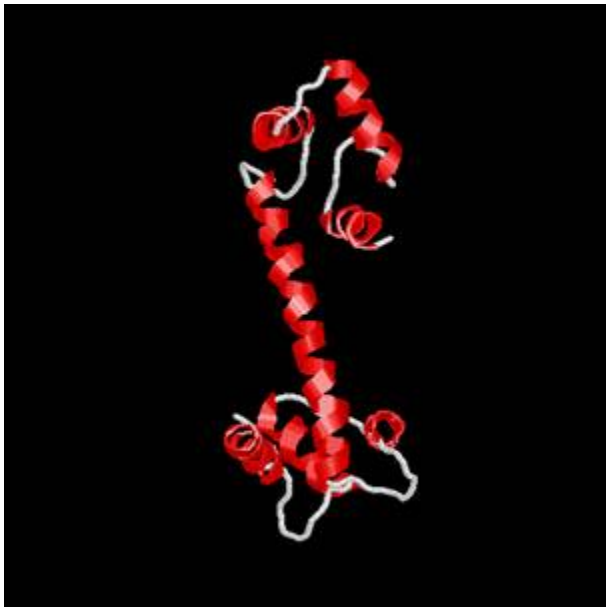


Figure 2.2: Calmodulin structure. Depiction of structures for calmodulin without calcium (apo-CaM, PDB 1CFD) and the calcium-saturated form (Ca_4: CaM, PDB 1CLL).

Human centrin 2 is a 172 amino acid protein with a molecular weight of 19,738 Da, the calcium binding sites (domains I and II) are located among position 41- 52 (I) and 150- 161 (II); it also possesses two ancestral calcium binding sites (domains III and IV) at the residues 77-88 (III) and 114- 125 (IV). This isoform of the protein has been involved in centrosome duplication and separation during each cell cycle (14).

The "knockout" of centrin 2 results in a failure of centriole duplication during the cell cycle in HeLa cells. Following inhibition of centrin 2 synthesis, the pre-existing pair of centrioles separate, and functional bipolar spindles form with only one centriole at each spindle pole. Centriole dilution results from the ensuing cell division and daughters cells are "born" with only a single centriole. These unicentriolar daughter cells may complete a second and even a third bipolar mitosis in which spindle microtubules converge onto unusually broad spindle poles and in which cell division results in daughter cells containing either one or no centrioles at all. Cells thus denuded of the mature or both centrioles fail to undergo cytokinesis in subsequent cell cycles, giving rise to multinucleated cells and finally die (1).

A conserved carboxyl-terminal region of centrin from diverse species includes a consensus motif for protein phosphorylation that is typical of serine/threonine kinases. It has been shown that in cultured vertebrate cells, centrin is phosphorylated early in mitosis near its carboxyl terminus at serine residue 170, when the newly duplicated centrosomes separate to give rise to the mitotic spindle poles. The spindle pole localization of phosphocentrin remains high until metaphase and then diminishes to basal levels by

telophase. The timing of centrin phosphorylation suggests that it may initiate the separation of duplicated centrosomes in preparation for mitotic spindle formation (6). More recently, aberrant centrin phosphorylation has been demonstrated in human breast tumors that have amplified centrosomes containing supernumerary centrioles and/or excess pericentriolar material (2). Figure 2.3

Like other proteins from the calcium binding family, centrins seem to act as Ca^{2+} sensor and they interact with specific target proteins to modulate their cellular activity. Sfi1p is one the centrosome target proteins for centrin that have been identified in the yeast, having a homologue in higher eukaryotes and in human; genetics studies had shown that SF1 is required for proper mitotic spindle assembly and duplication (15), suggesting that Cdc31p and Sfi1p interact functionally and play an important role in SPB duplication. Another centrosomal target protein identified in the yeast was Kar1p that has no mammalian homologue (16).

Analysis of the Sfi1p yeast sequence revealed a remarkable series of seventeen internal centrin binding repeats with the consensus motif $AX_7LLX_3F/LX_2WK/R$, with cluster of repeats separated by gaps of between 23 and 35 amino acids. Genome database mining identified candidate homologues with similar repeats in human and mice. Two versions of human Sfi1p (hSfi1p) were identified with almost identical sequences containing twenty three internal consensus repeats (17).

Recently, two potential centrin targets were identified outside the centrosome: the nuclear protein complex NER (nucleotide excision repair) (18) and the nuclear mRNA export machinery (19). In the NER process, Hcen 2 was found to be associated with the heterodimer formed by the XPC protein (xeroderma pigmentosum group C) and the hHR23B protein that was shown to recognize and bind to the damaged DNA. XPC has a seventeen residue fragment with high affinity for Hcen 2. (20).

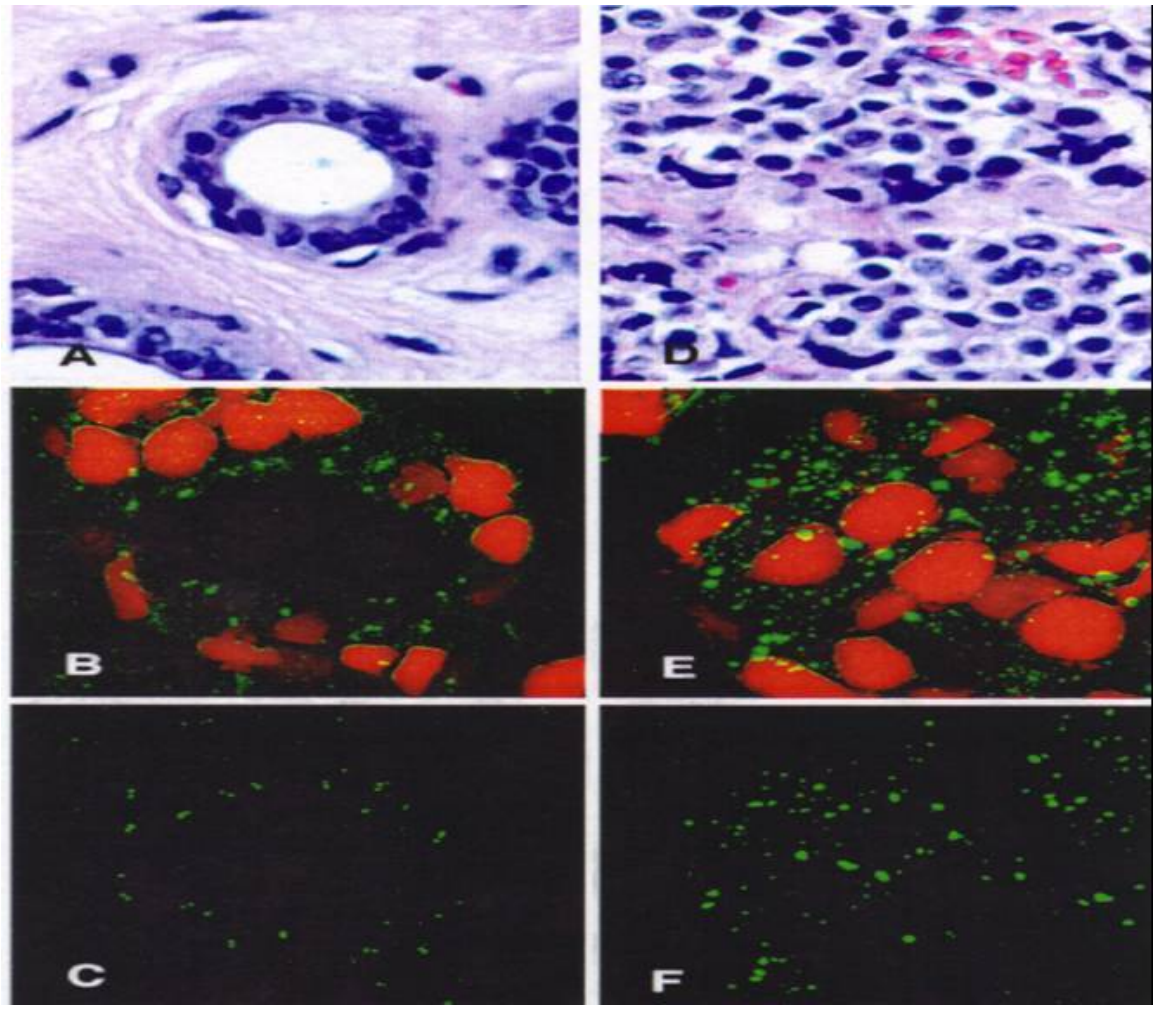


Figure 2.3: Centrosome number and size in normal breast cells and breast adenocarcinoma cells. Hematoxylin and eosin-stained sections of normal human breast duct (A) and breast adenocarcinoma (D). (B) confocal image of a normal breast duct satined for centioles with anticentrin mAb 20H5 (green) and for nuclear DNA with propidium iodine (red). (C) Binary processed image showing the volume of centrin labelin for the same normal epithelial image shown in B. (E) Confocal image of a breast adenocarcinoma stained as above. Many large centrin-stainig spots mark the location of abnormal centrosomes in the tumor tissue. (F) Binary processed image showing the volume of centrin labeling for the same tumor image shown in E.
Figure taken from Lingle, W. L. et all (1998) Proc. Natl. Acad. Sci. USA. 95, 2950-2955.

3 Materials and Methods

Transformation of *E. coli* BL21-Gold (DE3) competent cells

The lyophilized recombinant molecule pTP6 containing Hcetn2 cDNA coding sequence (Lee and Huang, 1993) in pT7-7 (generously supplied by Dr. Schiebel, from the Peterson Institute for Cancer Research, UK)(10) was reconstituted in 1 mL of sterile deionized water (18 MΩ) and its concentration and purity was determined by measuring its absorbance at 260, 280 and 320nm.

Transformation of the competent cells *E. coli* BL21-Gold (DE3) (from Stratagene, La Jolla, CA) was done in three separate reactions using Hcen2 in pT7-7, pUC18 (standard vector) and a third reaction without plasmid (negative control) following the protocol provided by Stratagene, based on the heat shock method (21). These transformation reactions were plated on LB agar media (Formulation/L: 10G Tryptone, 5g Yeast Extract, 5g NaCl, 15g Bacto Agar) containing 50 μg/mL of ampicillin (Sigma Chemical Company, St.Louis, MO) and incubated at 37^{o}C looking for isolated colonies of positive transformant cells.

The isolated colonies containing Hcen2 in pT7-7 construction were used to grow and overnight culture in LB broth (Formulation/L: 10G Tryptone, 5g Yeast Extract, 5g NaCl) with 50 μg/mL of ampicillin at 37^{o}C with vigorous shaking (250-300 rpm) and the growth culture was used to prepare cell stocks by adding 200 μL of glycerol and 800 μL of saturated culture. The stocks were stored at –80^{o}C.

3.2 Sequencing of DNA

To ensure that the cells were transformed with the appropriate recombinant cDNA molecule and that there was no contamination with another plasmid or any mutation in the centrin coding region, a plasmid extraction and cDNA sequence was done as follows:

A single fresh colony of *E.coli* BL21- Gold (DE3) pT7-7/Hcen2 was inoculated in 5 mL of LB broth containing 50 μg/mL of ampicillin and incubated for 8 hr at 37^{o}C with vigorous shaking (250-300 rpm); the starter culture was diluted in 100 mL of medium and grown at 37^{o}C for 12-16 hr with vigorous shaking.

Plasmid cDNA was isolated using the Qiagen plasmid Midi Protocol (QIAGEN Inc, Valencia, CA) following the protocol provided by the producer (22), the concentration and purity was measured by absorbance at 260, 280 and 320 nm. The purified plasmid was lyophilized and sent for sequencing to Mayo Clinic and Foundation, DNA Core Facility. The cDNA nucleotide sequence results were then compared to the Hcetn2 gene found in the GenBank from the National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) (23).

3.3 Small Scale Protein Expression and Amino Acid Sequencing

A single colony of *E. coli* BL21 pT7-7/Hcen2 was inoculated in 5 mL of LB broth supplemented with 50 μg/mL of ampicillin and incubated overnight at 37^{o}C with vigorous shaking. The overnight culture was inoculated into 95 mL of LB-ampicillin broth to obtain a 1:20 dilution. Cell density was monitored by measuring the OD of the

culture at 600 nm using a Jasco V-560 UV/Vis Spectrophotometer. Aliquots were collected at each measured point and stored -20^{o}C for further analysis.

An induction of centrin over expression was performed by adding 0.5 mM isopropylthio-β-galactoside (IPTG) (Sigma Chemical Company, St.Louis, MO) at mid logarithmic phase, and the culture was grown until it reached the stationary phase. Cells from the aliquots and final culture were harvested at 4 ^{o}C and resuspended in Laemmli sample buffer (24) (250 mM Tris, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 4 mM EDTA, 0.08% Bromophenol Blue, water) and lysed by sonication. These whole cell lysate samples were used to run on two separated 5% stacking 15% separating SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) under identical conditions.

One gel was stained with Coomasie Brilliant Blue (detection range 0.5-20 μg of protein) to reveal protein band with a migration pattern similar to pure *Chlamydomonas* centrin Mr ~ 20 kDa assumed to be Hcen2, the second gel was then used for semidry blotting using a PVDF membrane (Bio-Rad Laboratories, Hercules, CA) and stained with Coomasie Brilliant Blue similar to the gel protocol and sent for partial amino acid sequencing of the first 15 residues. The amino-terminal end sequencing procedure was performed at Mayo Clinic and Foundation Protein Core Facility. The chemical processes used in the N-terminal sequencing instruments are based on the sequential degradation reactions described by Pehr Edman in the 1950s.

The partial amino acids sequence results were then compared with the published protein sequence found in the the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) (www. expasy.org).

3.4 Large Scale Expression

Following an established protocol for Pastrana-Rios (4), the transformed competent cells were grown in 5 L of either 2xYT medium (Formulation/L: 16g Tryptone-B, 10g Yeast Extract-B, 5g NaCl, from Bio 101, Carlsbad, CA) or ^{13}C isotope enriched minimal media (Spectra Stable Isotopes, Columbia, MD) both containing 50 μg/mL of ampicillin and using the biorreactor Bioflo 3000 (New Brunswick Scientific, Edison, NJ) to obtain gram quantities of cell pellet. By the use of this bioreactor it was possible to monitor and control the pH, temperature, agitation and dissolved O_2 concentration, to ensure the best performance.

The growth rate was monitored by optical density at 600 nm, every 30 minutes; and when the growth curve reached the middle of the logarithmic phase, 0.5 mM IPTG was added to induce the over-expression of the desired protein. The culture was grown until it reached the stationary phase. The bacterial cells were harvested by centrifugation at 3,500 rpm for 20 minutes at 4 ^{o}C using a BECKMAN J2-MC centrifuge (BECKMAN Instruments, Palo Alto, CA) and the pellet cells were stored at -80^{o} C until the purification process was done.

Samples of the bacterial culture aliquots from before and after IPTG induction were also harvested and the pellet cells were resuspended in Laemmli sample buffer, sonicated with a micro probe Ultrasonic Homogenizer 4710 series (Cole Parmer, Vernon Hills, IL) at maximum power for three to five 30 seconds pulses and 1min cooling period while on ice until the mixture looked free flowing and no longer viscous.

Again these whole cell lysates samples were used to run a 5% stacking 15% seperating SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel), stained with Coomasie Brilliant Blue to verify that in fact there was centrin over-expression in those cells before carrying out the purification process.

3.5 Purification of the Protein by Affinity Chromatography

Following a modification of purification protocols developed by Pastrana-Ríos *et al* and Baron *et al* (6, 25) the cell pellet frozen or otherwise, was thawed in four times the amount (w/v) of cold (4 ^{o}C) cell lysis buffer (50 mM Tris, 0.5 mM EDTA, 0.5M NaCl, 0.1% IGPAL, 0.04% NaN_3) containing a protease inhibitors cocktail (2.0 mg/mL aprotinin, 0.5 mg/mL leupeptin, 1.0 mg/mL pepstatin A), then sonicated in an ice bath with a macro probe Branson sonifier 450 (Branson Ultrasonics Corporation, Damburu, CT) to further break the cells and sheer the DNA. The sonication process was done at maximum power for three to five 30 seconds pulses and 1 minute resting period while on ice. This process was done until the lysate was cream colored and free flowing (low viscosity).

The whole cell lysate was centrifuged at 10,000 rpm using a BECKMAN J2-MC centrifuge with JA-20 rotor (BECKMAN Instruments, Palo Alto, CA) for 20 minutes at 4 ^{o}C, obtaining supernatant (S_1) and pellet (P_1). The P_1 was discarded after taking a small sample of both S_1 and P_1 for SDS-PAGE and troubleshooting. The supernatant S_1 was recovered and 2 mM $CaCl_2$ and 4 mM $MgCl_2$ were added followed by a second centrifugation at 31,000 rpm using a BECKMAN L-80 Ultracentrifuge with T1-80 rotor (BECKMAN Instruments, Palo Alto, CA) for 1 hour at 4 ^{o}C, again obtaining supernatant (S_2) and pellet (P_2) fractions. S_2 was kept and P_2 was discarded after taking samples of both S_2 and P_2 for SDS-PAGE.

The Phenyl-Sepharose Cl-4B (Sigma, St louis, MO) was packed and equilibrated with eluant A (50 mM Tris, 0.5 M NaCl, 4.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 0.04% NaN_3, 0.1% IGEPAL) with a flow rate 2.7 mL/min, until a stable baseline at A_{280} was achieved. The supernatant sample S_2 was filtered through a 0.22 μm filter (Nalgene, Rochester, NY) to remove any debris before applying the sample to the column.

The filtrate was then applied to the Phenyl-Sepharose column with CL-4B eluant A and the flow-through (named void A) was collected until baseline returned to zero. After that, centrin and another calcium binding protein were eluted and collected with CL-4B eluant B (50 mM Tris, 0.5 mM EDTA, 5 mM EGTA, 4.0 mM $MgCl_2$, 0.04 % NaN_3) until baseline returned to zero. Finally, any remaining proteins hydrophobically bound to the matrix were eluted and collected with CL-4B eluant C (50 mM Tris, 0.5 mM EDTA, 5 mM EGTA, 0.04 % NaN_3) until baseline returned to zero.

At the end of the process, the column was washed for next use by eluting any tightly bound protein to the matrix with 3 bed volumes of 30% aqueous isopropanol, rinsed with 3 bed volumes of deionized water and either equilibrated with 3 bed volumes of CL-4B eluant A before another chromatographic run or 20% ethanol for long term storage.

Centrin containing fractions were identified by 15% SDS-PAGE and Bradford assay (26). All the fractions containing centrin were pooled, concentrated and re-equilibrated in ion exchange buffer A (40 mM Tris, 2 mM $CaCl_2$, and 2 mM DTT at pH 7.4) for the next purification step or in protein buffer (50 mM Hepes, 150 mM NaCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$) for freeze dry storage.

The chromatographic parameters used were: flow rate 2.17 mL/min, absorbance of 280 nm, AUFS of 0.5, chart speed of 1 cm/h.

3.6 Anion Exchange Chromatography

A 5 ml Econo-Pac High Q cartridge (Bio-Rad, Hercules, CA) was prepared following the protocol supplied by the manufacturer (27). Sample protein concentration was adjusted to 1-10mg/mL or more with a maximum sample buffer of 5 mL and the buffer was change to ion exchange buffer A (40 mM Tris, 2 mM $CaCl_2$, and 2 mM DTT, 0.08% NaN_3 at pH 7.4).

The sample was loaded on the column and eluted with ion exchange buffer A for 3 minutes. Centrin was eluted from column with a NaCl linear increasing gradient. Several gradients were tried including: 0-1 M NaCl, 0-0.75 M NaCl, 0. 5M NaCl and 0-0.35 M NaCl, through a progressive linear mixture of ion exchange buffer A and B (40 mM Tris, 2 mM $CaCl_2$, and 2 mM DTT, 0.08% NaN_3, different NaCl concentration at pH 7.4) . At the end of the ionic gradient, the column was washed of other absorbed proteins with ion exchange buffer C (40 mM Tris, 0.08% NaN_3, 2 mM NaCl at pH 7.4).

The centrin containing fractions were identified by 15% SDS-PAGE and Bradford assay (26). All the fractions containing centrin were pooled, concentrated and re-equilibrated in protein buffer (50 mM Hepes, 150 mM NaCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$) for freeze dry storage and calorimetric studies.

The chromatographic parameters used were: flow rate 2.0 mL/min, absorbance of 280 nm, AUFS of 0.2, chart speed of 12cm/h. The methods used are summarized in Figure 3.1.

3.7 Differential Scanning Calorimetry (DSC)

The calorimetric studies were performed with a digitally controlled VP-DSC microcalorimeter from MicroCal. All samples buffer were changed to protein buffer. Protein concentration was determined from the ultraviolet absorption spectrum at 274 nm using a Jasco V-560 UV/Vis spectrophotometer after base line correction by the Microsoft Windows-based software package Origin 7 (Microcal Software, Inc,

Northampton, MA). The molar extinction coefficient of the tyrosine at 274 nm was calculated to be 1309.9 M^{-1} cm^{-1} in the presence of calcium.

All experiments were carried-out at a constant pressure (~25 psi) to prevent possible degassing of the solution during heating. The thermal perturbation of the protein was done by increasing the temperature from 10-127^{o} C, scan rate 60 deg/h.

Calculation of the melting temperature (T_m) was performed using the DSC curves after, subtracting the appropriate baselines, normalizing the data for protein concentration and correcting the transition for the difference in the heat capacity between the initial and final states using a Microsoft Windows-based software package Origin 7.

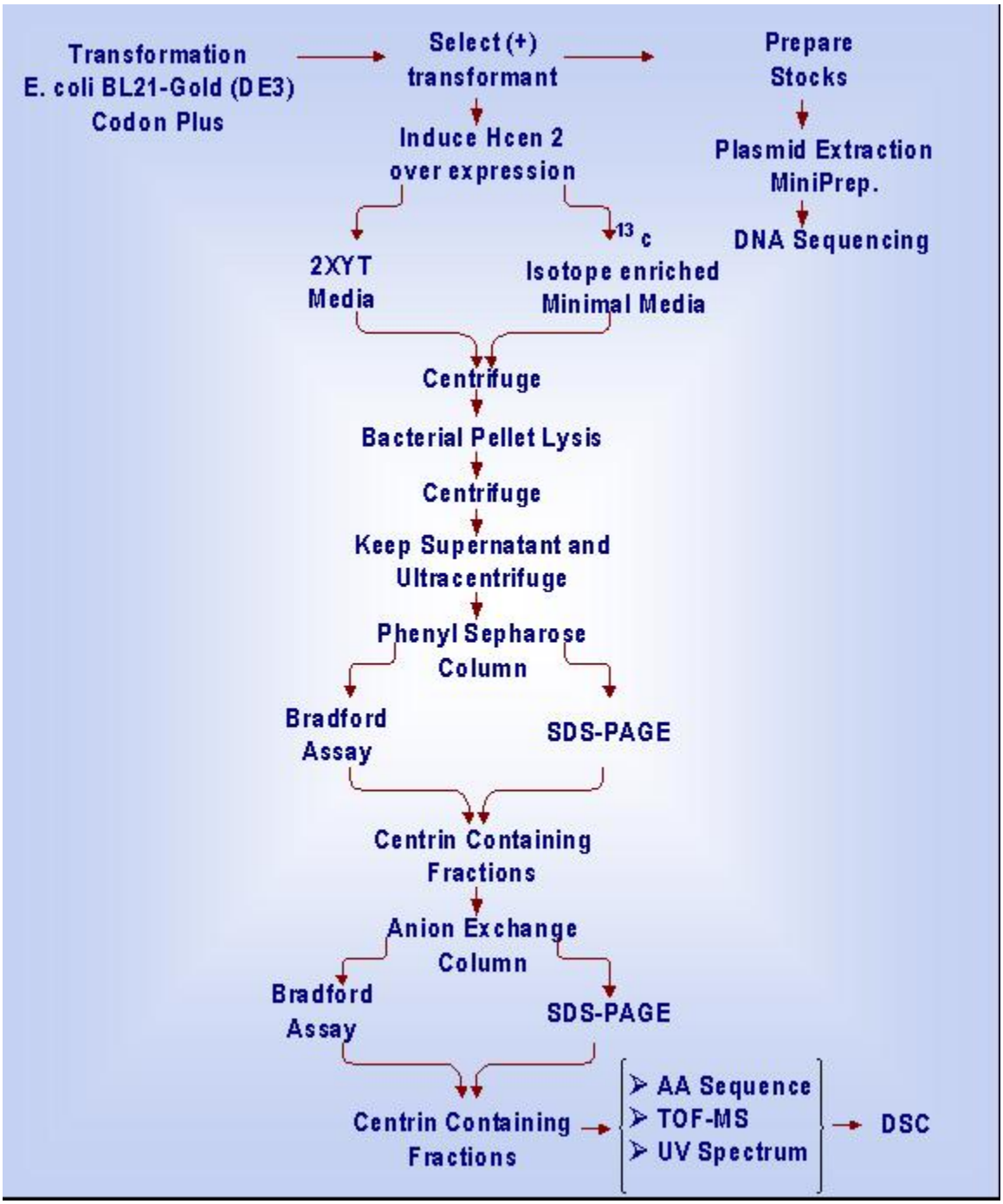


Figure 3.1: Schematic representation summarizing the purification protocol used for Hcen2

4 Results

A positive transformation was achieved in the cell transformed with pTP6 (Hcetn2 in pT7-7) molecule and pUC 18 (standard vector), and healthy isolated colonies were observed without sings of contamination; no cell growth was observed on the plate spread with the cells treated without plasmid. Since *E. coli* BL21-Gold (DE3) codon plus competent cells do not have ampicillin resistance gene, only cells transformed with a plasmid conferring ampicillin selectivity are able to grow on LB-plates with ampicillin. The recombinant molecule pTP6 carries Hcetn2 coding region located between the Nde I and Sal I restriction sites of the expression vector pT7-7, which carries an ampicillin resistance gene.

4.1 DNA and Amino Acid Sequence Results

100 µl of pTP6 molecule were obtained after the plasmid purification protocol and its concentration and purity were calculated as follows:

The DNA concentration determination and purity was assessed by UV spectroscopy. Since, the absorptivity of the DNA is known to be 50 µg/mL per absorbance unit at 260 nm and the absorbance ratio R 260/280 provides the purity index. The greater the ratio, the lower the protein contamination (absorbance at 280 is used to determine the presence of protein) in the sample.

Plasmid containing solution absorbance at 260 nm was 0.2749 and at 280 nm was 0.1359 with a 260/280 ratio of 2.0, which indicates that this is a highly pure plasmid preparation. These absorbance values were used to calculate plasmid concentration in

solution as shown in Equation 4.1. This pTP6 recombinant molecule was sequenced at Mayo Clinic and Foundation DNA Core Facility to verify the presence of the Hcetn2 cDNA.

Equation 4.1: pTP6 plasmid concentration calculation

$[DNA] = A_{260}$ x 50 µg/mL x dilution factor

$[DNA] = 0.2749$ x 50 µg/mL x 1

$[DNA] = 13.745$ µg/mL

HCetn2 in plasmid pT7-7

pT7-7 vector 5'end

AAGATCTTC CTATGGTGCA CTCTCAGTAC AATCTGCTCT GATGCGCTAC
GTGACTGGGT CATGGCTGCG CCCCGACACC CGCCAACACC CGCTGACGCG CCCTGACGGG
CTTGTCTGCT CCCGGCATCC GCTTACAGAC AAGCTGTGAC CGTCTCCGGG AGCTGCATGT
GTCAGAGGTT TTCACCGTCA TCACCGAAAC GCGCGAGGCC CAGCGATTCG AACTTCTGAT
AGACTTCGAA ATTAATACGA CTCACTATAG GGAGACCACA ACGGTTTCCC TCTAGAAATA
ATTTTGTTTA ACTTTAAGAA GGAGATATA***C AT***

Hcen2 coding region

<u>*ATG*</u> GCCT CCAACTTTAA GAAGGCAAAC
ATGGCATCAA GTTCTCAGCG AAAAAGAATG AGCCCTAAGC CTGAGCTTAC TGAAGAGCAA
AAGCAGGAGA TCCGGGAAGC TTTTGATCTT TTCGATGCGG ATGGAACTGG CACCATAGAT
GTTAAAGAAC TGAAGGTGGC AATGAGGGCC CTGGGCTTTG AACCCAAGAA AGAAGAAATT
AAGAAAATGA TAAGTGAAAT TGATAAGGAA GGGACAGGAA AAATGAACTT TGGTGACTTT
TTAACTGTGA TGACCCAGAA AATGTCTGAG AAAGATACTA AAGAAGAAAT CCTGAAAGCT
TTCAAGCTCT TTGATGATGA TGAAACTGGG AAGATTTCGT TCAAAAATCT GAAACGCGTG
GCCAAGGAGT TGGGTGAGAA CCTCGATGAT GAGGAGCTGC AGGAAATGAT TGATGAAGCT
GATCGAGATG GAGATGGAGA GGTCAGTGAG CAAGAGTTCC TGCGCATCAT GAAAAAGACC
AGCCTCTATT <u>AA</u>

3'end sequence

GATCAGTG TCTTCTTTTT CTACTGCAAG CACATGTAAC

pT7-7 vector 3'end

GTCGAGTATA
TAACTACGAT ACGGNAGGGC TTACACATCT GGCCCCAGTG CTGCANTGAN ACCGCGAGAC
CCACGCTCAC GCGGCTCCAG ATTTATNANC AATAAACCAN CCCCGCCGGA NGGGCCTAGT
CGCANANGTG GTCCTGGCAA CTTTATCCGN CCTCCACCCA NTTTATTAAT TTGTTGCCCN
GGTAAGCTAN AGTAAGTAGT TCCNCCNGTT NATANTCTNG GCAACGTTTT TGCCATNCNT
TACAGGNTTN GTGGTGNCCC

Figure 4.1: pTP6 recombinant molecule DNA sequence. These sequence results show some nucleotides from the vector flanking the 5' end and 3' end of the Hcetn2 gene. It also shows the complete nucleotide sequence of Hcetn2 coding region (bold) including the location (underline) of the start and end transcription codons, as well as the Nde I (italic and blue) and Sal I (italic and red) restriction sites. DNA sequencing was performed at Mayo Clinic and Foundation DNA core facility.

The DNA sequencing results are shown in Figure 4.1 including 321 nucleotides of the vector flanking the 5' end of the Hcetn2 coding region and 270 nucleotides at the 3' end. More importantly, these results also show the 519 nucleotides corresponding to Hcetn2 coding sequence beginning with an ATG start codon which is also part of the Nde I restriction site and 39 nucleotides corresponding to the 3' end sequence of the gene located just after the stop codon. The Sal I restriction site as expected is located outside of the cDNA insert (Hcetn2) sequence.

A comparison was done with the Hcetn2 gene (12) found in the GenBank from the National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST), and as expected it showed a ninety nine percent match among these two sequences with an open reading frame starting with ATG encoding 172 amino acids polypeptide with a predicted molecular weight of 19,738 Daltons and ending with TAA stop codon (23) (*data shown in appendix A*).

The growth pattern of 100 ml of pTP6 transformed *E. coli* is shown (Figure 4.2). This bacterial culture was grown in a fermbach flask using an orbital shaker at 250 rpm as agitation rate and the only controlled parameters were temperature and agitation while monitoring optical density at 600 nm. In these cells Hcen2 protein over expression was achieved by adding IPTG during the mid log phase.

As demonstrated in Figure 4.3, centrin a protein ~ 20 kDa shows no expression or very low basal level expression before induction with IPTG and is over expressed after

induction. This phenomenon is due to the fact that *E. coli* BL21-Gold (DE3) codon plus competent cells carry a copy of the T7 bacteriophague gene 1 (which codifies for the T7 RNA polymerase) under control of the *lacUV5* promoter. On induction with IPTG, the *lacUV5* promoter is derepressed, allowing over expression of T7 RNA polymerase. On the other hand, pT7-7 plasmid contains a T7 RNA polymerase promoter upstream of the Hcetn2 gene. These two characteristics allow controlled over-expression of the T7-promoted target Hcetn2 gene from the pT7-7 vector, because T7 RNA polymerase is highly selective for initiation at its own promoter sequences and it does not initiate transcription from any sequences in the *E. coli* DNA. Figure 4.4

Two SDS -PAGE were carried-out under identical conditions for the whole cell lysate obtained from the induced transformed bacterial cells. One gel was stained with Coomasie Brilliant Blue to reveal protein band with a migration pattern similar to pure *Chlamydomonas* centrin Mr ~ 20 kD assumed to be Hcen2, the second gel was then used for blotting using a PVDF membrane, stained with Coomasie Brillian Blue similar to the gel protocol and sent for partial amino acid sequencing. The amino terminal sequence results are shown in Table 4.1. The partial amino acid sequence resulted in positive assignments as compared to wild type Hcen 2 amino terminal sequence found in the Swiss-Protein Data Bank with PDB number P41208.

The amino terminal amino acids are the most distinctive and variable feature among centrins from various species and among human centrins which are at least 80% identical in amino acid sequence, with most of the differences occurring in the first amino

acid. These results in conjunction with a hundred percent match in cDNA sequence suggest that the expression of Hcen 2 has occurred with no mutations.

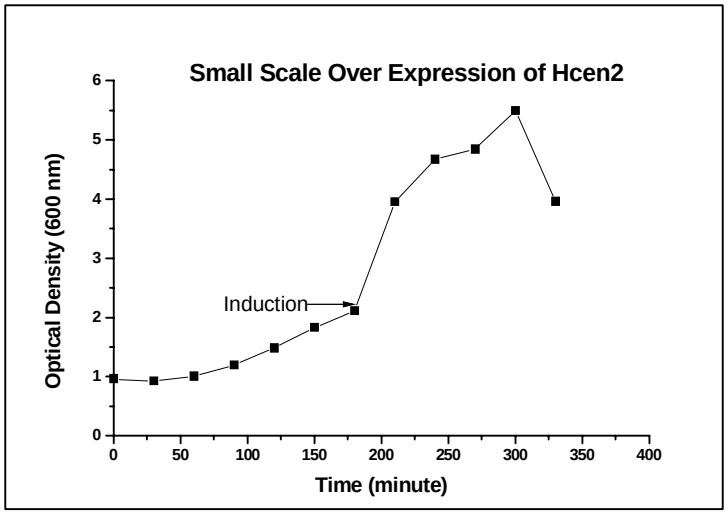


Figure 4.2: Growth curve of positive transformants *E. coli* BL21-Gold cells. These cells were grown in LB-broth media with ampicillin in a fermbach flask at 37 ^{o}C until they reached the stationary phase. Isopropylthio-β-D-galactoside (IPTG) was added at mid-log phase (180 min) to induce the synthesis of the desired recombinant protein.

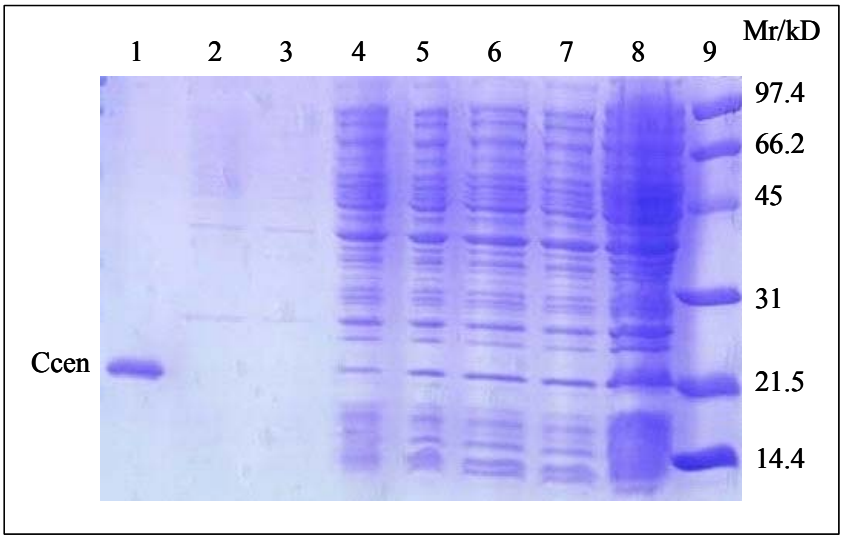


Figure 4.3: Small Scale Induction SDS-PAGE 5% stacking- 15% separating. The proteins bands corresponding to a pTP-6/ *E. coli* whole cell lysate shows no basal level expression of HCen2 at pre-induced 60 and 120 minutes (lanes 2 & 3 where we can see basal level expression of E. coli somatic proteins) and high level expression after the induction times 240, 270, 300, 330 and 360 min (lanes 4, 5, 6, 7 & 8, respectively) as compared with the migration pattern of pure *Chlamydomonas* centrin (Ccen) in lane 1. Lane 9: SDS-PAGE Bio Rad low range standard.

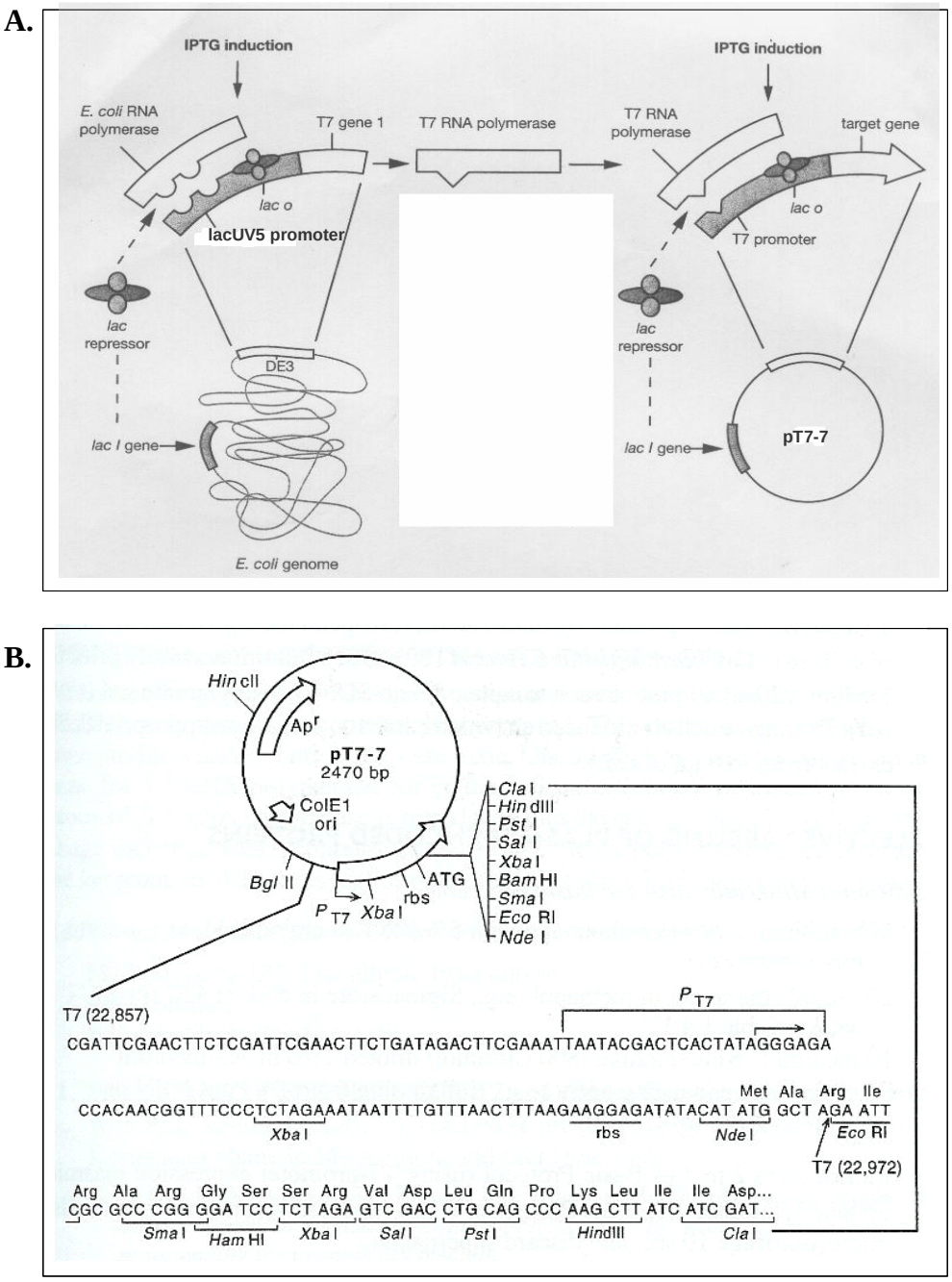


Figure 4.4: T7 RNA polymerase/promoter system for specific gene expression. (A) Host and vector elements that are available for control of T7 RNA polymerase levels and subsequent transcription of a target gene (*Hcetn2*) cloned in a pT7-7 vector. The T7 RNA polymerase is under the control of the *lacUV5* promoter, which allows low levels of transcription in the uninduced states. (B) shows pT7-7 cloning vector that contains a T7 promoter, the gene encoding resistance for ampicillin and the ColE1 origin of replication, also is shown the start codon (ATG) upstream of the polylinker sequence. *Modified from Tabor, S; and Richardson, C. C. (1984) Proc. Natl. Acad. Sci. USA. 82, 1074-1078.* (28)

Table 4.1: Summary of Amino Acid Sequence Results

Cycle no	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
N-terminal analysis Hcen2	A	S	N	F	K	K	A	N	M	A	S	S	S	Q	R
N- terminal analysis ^{13}C Hcen2	A	S	N	F	K	K	A	N	M	A	S	S	S	Q	R
*Hcen2 Published Sequence	A	S	N	F	K	K	A	N	M	A	S	S	S	Q	R

* Swiss-Prorein Data Bank (www.expasy.org) Accession number P41208.

4.2 Large Scale Overexpression

The fermentation profiles for large scale culture where the growth rate, pH, temperature and dissolved oxygen were recorded and analyzed from four different Hcen 2 positive transformant *E. coli* colonies grown in either 2xYT media or ^{13}C isotope enriched minimal media (Spectra Stable Isotopes, Columbia, MD). The culture grown in 2xYT media was supplemented with glucose as a major carbon source, glucose infusion was started just after the induction point.

These data were compared to analyze differences in pellet cell weight and protein over expression (Figure 4.5). There were huge differences in culture yield between cells grown in 2xYT-broth and the one grown in ^{13}C isotope enriched minimal media, the later result in a maximum optical density of only 1.5 and cell pellet weight of 5.9 g (Figure 4.5b) as compared with the ones grown in 2xYT which yield a maximum optical density of 14.64 and a pellet weight of 71.49 g (Figure 4.5a); this difference is due to the fact that the glucose infusion results in a constant carbon source that supports the aerobic growth and a maximum performance of the culture.

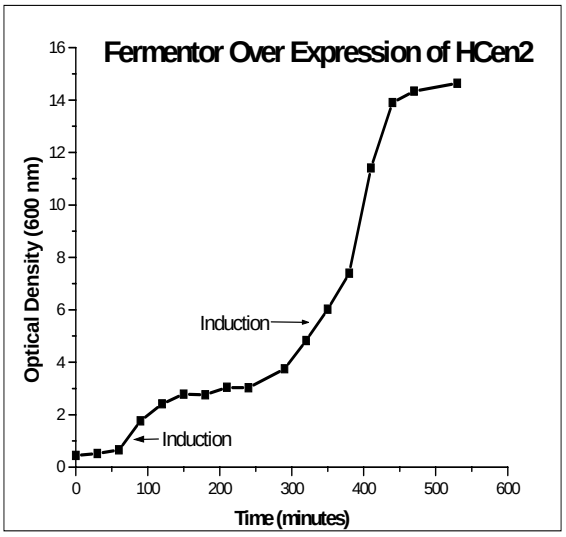
In another instance there were noted some differences were observed between the three cultures grown in 2xYT broth. As it can be seen in Figure 4.4a, this culture resulted in a maximum output with the most physiologic and predictable growth curve, and this can be explained because this was the only culture grown from a fresh plated pTP6/ *E. coli* BL21-Gold (DE3) codon plus competent cells colony, whereas the other two were grown from a frozen stock.

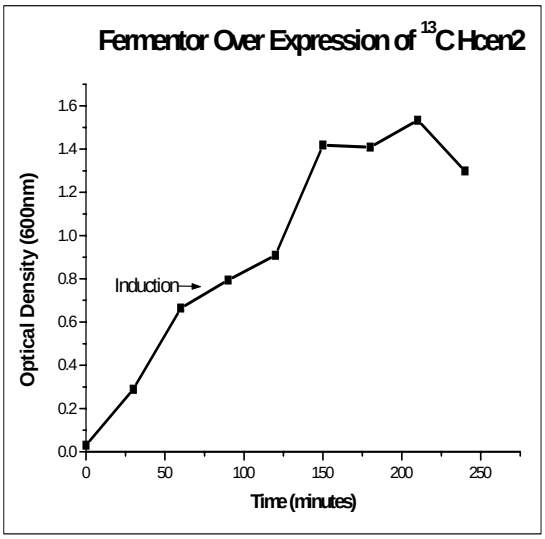
The culture shown in Figure 4.5c was the one with poorest yield not only because the source of the initial inoculums, but also because there was an oxygen leak and the dissolved oxygen measurement were below 80% at post-induced times. This caused a rapid switch from the log phase to the death phase without entering a stationary phase. Finally, the growth curve shown in Figure 4.5d showed an intermediate pattern.

It was possible to induce protein over expression in all four fermentor runs with the same concentration of IPTG, and there was no increase in protein expression by the addition of IPTG one or two times during early-mid log phase (Figure 4.5).

a. Pellet weight: 71. 49 g
Initial OD_{600} 0.4

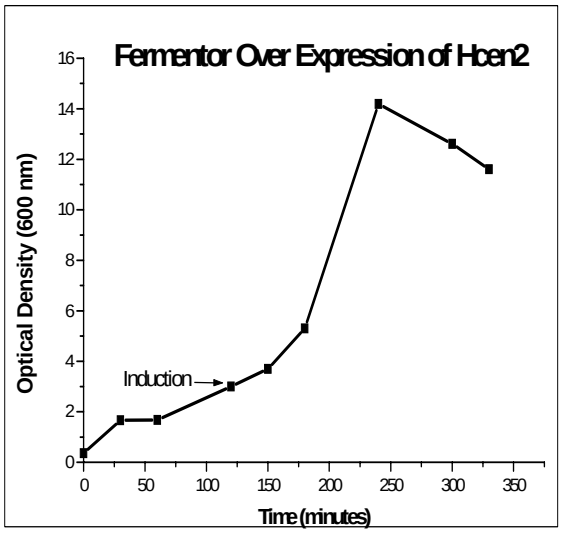
b. Pellet weight: 5.9 g
Initial OD_{600} 0.03





c. Pellet weight: 27 g
Initial OD_{600} 0.3

d. Pellet weight: 59.57 g
Initial OD_{600} 0.4



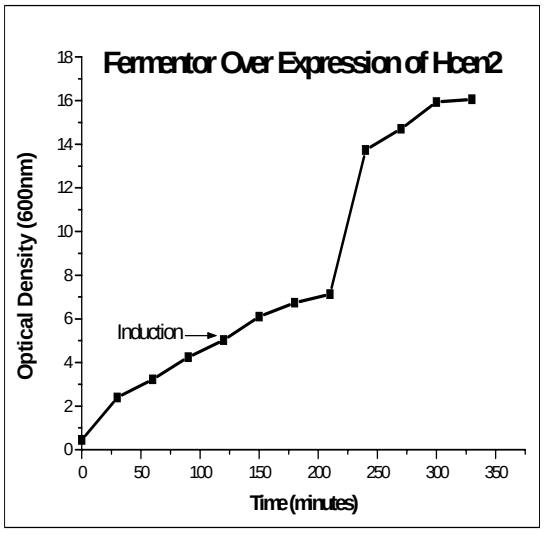
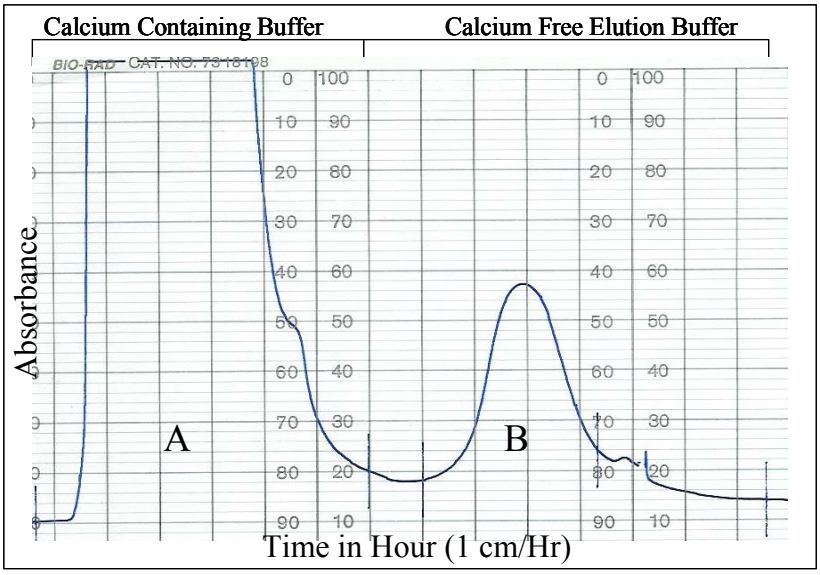
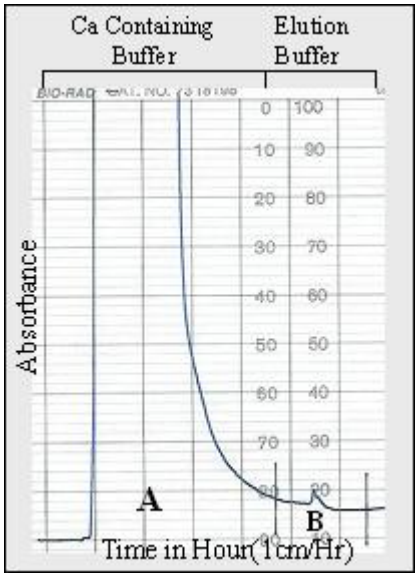


Figure 4.5: Growth profiles of four different fermentors running over expression. *E. coli* positive transformant for Hcen 2 were grown using either 2xYT or ^{13}C isotope enriched minimal media. The exact induction point is shown for each curve as well as the exact cell pellet weight.

4.3 Protein Purification by Affinity Chromatography





Figures 4.6 and 4.7: Phenyl-Sepharose Column Chromatograms for Hcen 2 and ^{13}C isotope labeled Hcen 2. Show the calcium dependent elution properties of centrin. During peak A elute proteins and others molecules that do not interact with phenyl-sepharose matrix and centrin elutes during the last half of peak B (fractions50-87) and the small decline to base line (fraction 88-110). The running parameters were as follows; flow velocity: 2.17 ml/ min, paper velocity: 1 cm/hr and 0.2 AUFS sensitivity. Each square has 1 cm^2 area.

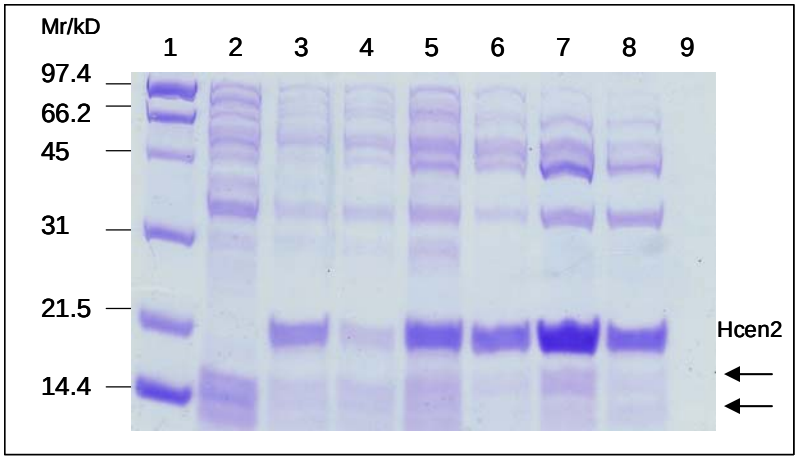


Figure 4.8: SDS-PAGE 15% separating. Samples corresponding to Phenyl Sepharose column fractions. Lane 1: SDS-PAGE Bio Rad low range standard, lane 2: void B fractions 1-47 (first half of peak B in Figure 4.5), lane 3, 4, 5, 6: void B Fractions 50, 60, 70 respectively, lane 7: fraction 88-100 (small notch in peak B, Figure 4.5), lane 8: fractions 101-110 (small decline to base line), lane 9: void volume C. Double arrows indicate duplets image of Hcen 2 proteolitytic cleavage products. Lanes 3-8 show duplet images of full length Hcen 2 (~ 20 kD) and Hcen 2 cleavage product (~ 18 kD).

Human centrin 2 was partially purified by affinity chromatography taking advantage of its hydrophobic interaction with Phenyl Sepharose CL-4B matrix in the presence of calcium and was eluted by the removal of calcium, suggesting that centrin, similar to calmodulin, suffers a change in conformation upon calcium binding which lead to the exposure of hydrophobic amino acids side chains.

The affinity purification of centrin can be divided in three main steps, as shown in Figures 4.6, 4.7 and 4.8. The first step consist of centrin binding to the surface of the matrix in the presence of reasonable low concentration of salts and cations (0.5 M NaCl and 2.0 mM $CaCl_2$ in eluant A), whereas other *E. coli* proteins that do not have affinity with the matrix eluted during peak A of the chromatographic record (Figures 4.6 and 4.7)

and are visible as more than 10 different proteins bands in the SDS-PAGE (lane 2 in Figure 4.8).

The second phase is centrin elution upon removal of calcium by the addition of chelating agents and the absence of calcium in eluant B (50 mM Tris, 0.5 mM EDTA, 5 mM EGTA, 4.0 mM $MgCl_2$, 0.04% NaN_3). This results in a loss of the hydrophobic interaction with the matrix and its elution during the second half of peak B in Figures 4.5 and 4.6. It was also shown that others proteins were eluted with centrin in void volume B fractions (Figure 4.8, lanes 3-8). The third phase consisting of cleaning elution showed no centrin presence indicating that wash with eluant buffer B was enough to elute centrin.

The small aliquots named Pellet 1 (P_1) and Pellet 2 (P_2) were also analyzed by SDS-PAGE, which were shown to have little amount of centrin. This pellet was comprised mainly of contaminating proteins and degraded components of cellular membranes, as assumed by its viscous nature. On the other hand the corresponding centrin's band was present in supernatant 1 and 2 as predicted because centrin is a highly soluble protein.

4.4 Protein Purification by Anion Exchange Chromatography

In a different set of experiments, centrin showed different elution pattern depending on variations of the ionic strength used in the elution buffer, whereas the pH and the final volume were kept at constant values to not affect proteins stability and solubility. When a high linear ionic gradient (0-0.75 M NaCl) was used, centrin

purification was not achieved; but it was able to eliminate a high percent of contaminating proteins in solution (peaks A, B, C shown in Figure 4.9); centrin was found in fractions 9-12 eluting from 0.3 to 0.4 M NaCl (peak D in Figure 4.9 and SDS-PAGE in Figure 4.10).

When an intermediate linear ionic gradient (0-0.5 M NaCl) was used, centrin still was not completely purified, but a better resolution between different proteins separation was achieved when compared with the high linear ionic gradient, which lead to a cleaner centrin preparation. Symmetric peak D from Figure 4.9 was replaced for a more resolved asymmetric peak, as shown in Figure 4.11 (C, D, E and F) and 4.12.

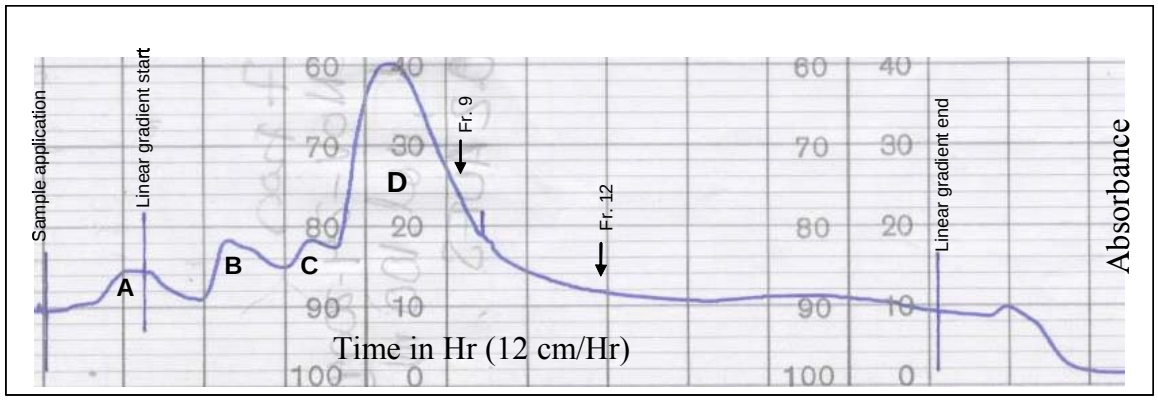


Figure 4.9: Ion Exchange Column Chromatogram with a 0-0.75 M ionic gradient. Centrin elution fractions are indicated by arrows. The running parameters were as follows; volume: 100 mL, flow velocity: 2 mL/min, paper velocity: 12 cm/hr and 0.2 AUFS sensitivity. Each square has 1 cm^2 area.

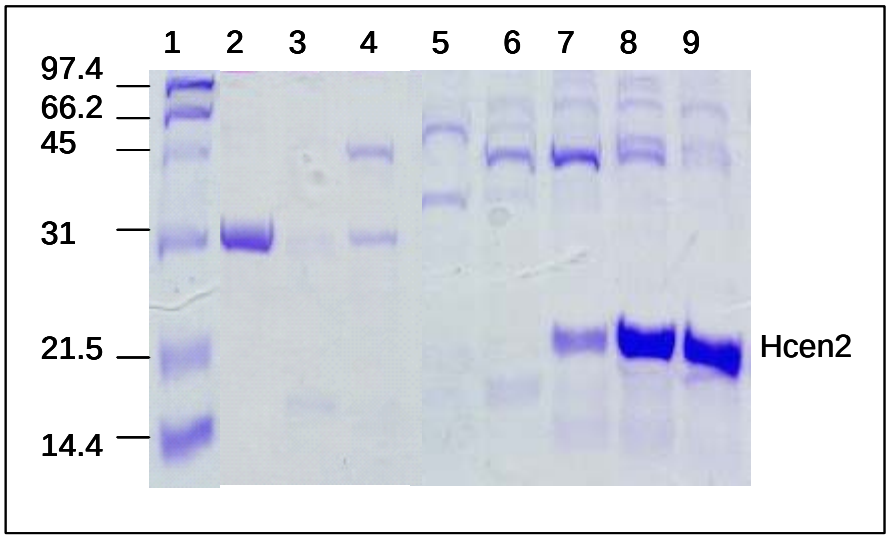


Figure 4.10: 15% SDS-PAGE of fractions from Ion Exchange Chromatography (high ionic gradient). Lane 1: SDS-PAGE Bio Rad low range standard, Lane 2-6: fractions 2, 4, 5, 6 and 7 respectively, lane 7-9: fraction 8, 9 and 12 respectively.

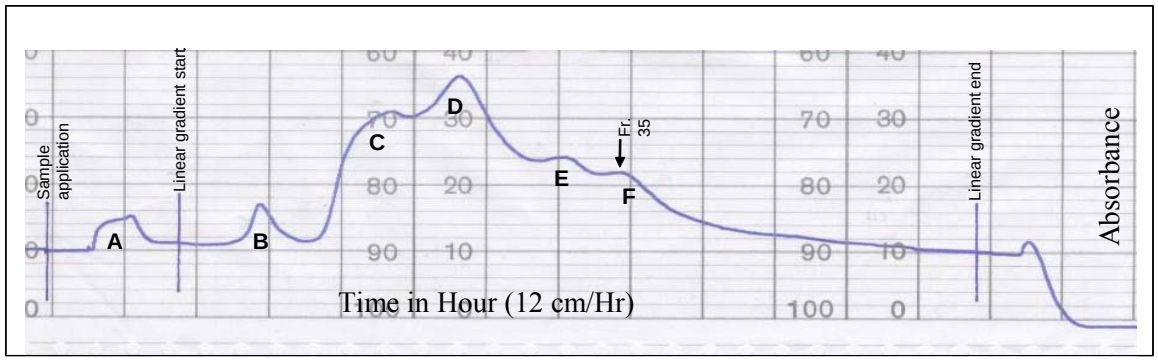


Figure 4.11: Ion Exchange Column Chromatogram with a 0-0.5 M ionic gradient. Centrin elution fraction is indicated by an arrow. The running parameters were as follows; volume: 100 mL, flow velocity: 2 mL/min, paper velocity: 12 cm/hr and 0.2 AUFS sensitivity. Each square has 1 cm^2 area.

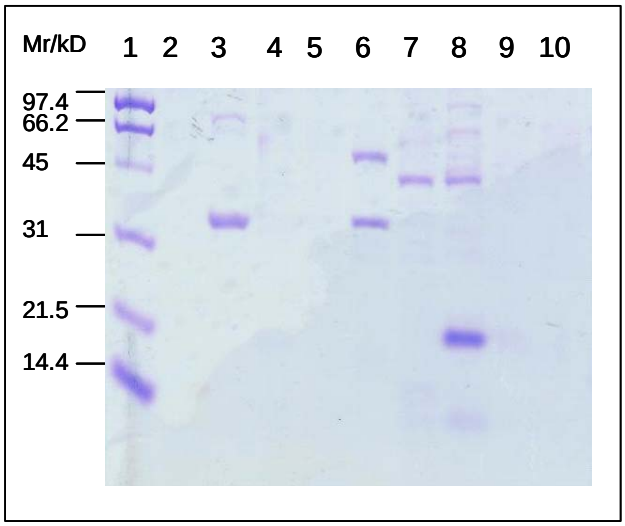


Figure 4.12: 15% SDS-PAGE of fractions from Ion Exchange Chromatography (intermediate ionic gradient). Lane 1: SDS-PAGE Bio Rad low range standard, lane 2: fraction 3, lane 3: fraction 11, lane 4: fraction19, lane 5: fraction 21, lane 6: fraction 26, lane 7: fraction 31, lane 8: fraction 35 (peak F in Figure 4.10) lane 9 & 10: fractions 54 and 62, respectively.

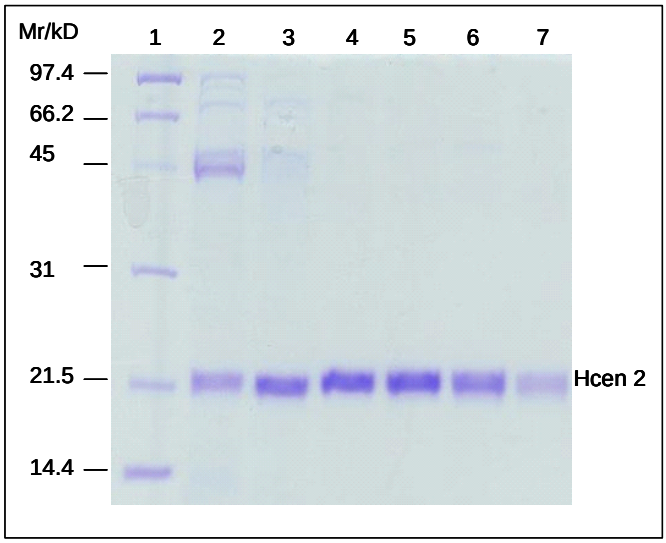


Figure 4.13: 15% SDS-PAGE of fractions from Ion Exchange Chromatography (high ionic gradient). Lane 1: SDS-PAGE Bio Rad low range standard, Lane 2-7: fractions 11 to17, respectively.

These results lead to the need for a second round of ion exchange chromatography of the partially purified centrin containing fractions from high and intermediate linear ionic gradient, using again either a high or intermediate linear ionic gradient with equal efficacy to purify *Human* centrin 2. Figure 4.13 shows that during first two fractions there still some contaminating proteins, but then there is a high purity elution of Hcen 2.

4.5 Purity and Identification of Spontaneous Cleavage Products of Hcen 2

Human centrin 2 was at least 99% pure as judge by SDS-PAGE and the characteristic UV spectra, which showed the absence of tryptophan absorbance at 280 nm, this could be used as purity criteria since centrin does not contain any tryptophan. In this spectra, it was also seen the characteristic phenylalanine absorbance at 257.4 nm and tyrosine absorbance at 274.6 nm (Figure 4.14).

The wild type Hcen 2 is prone to spontaneous proteolysis, resulting in the appearance of a consistence protein band ~ 19,000 Daltons and two occasional bands around 14,000 and 11,000 Daltons in SDS-PAGE. The action of the unidentified protease results evident sometimes just after Phenyl Sepharose purification or late after the first ion exchange purification. These bands were analyzed by N-terminal amino acid sequencing showing the persistent loss of the first 12 amino acids (shown in red and underline in Figure 4.15, resulting in a final molecular mass/z of 18,367 by Matrix assisted laser desertion ionization/time of flight (MALDI-TOF) mass spectrometry with the conservation of the four EF-hand motifs (Figure 4.16a). Hcen2 was also prone to cleavage around 71 and 83 peptide bonds.

^{13}C isotope labeled Hcen 2 look like a more resistance protein and its integrity evaluated by Matrix assisted laser desertion ionization/time of flight (MALDI-TOF) mass spectrometry yield a molecular mass/z of 20,425, in contrast, with the expected molecular mass of 20,595 Daltons resulting from the increase in the mass of the 857 ^{13}C over the 19,738 Daltons in the wild type protein (Figure 4.16b).

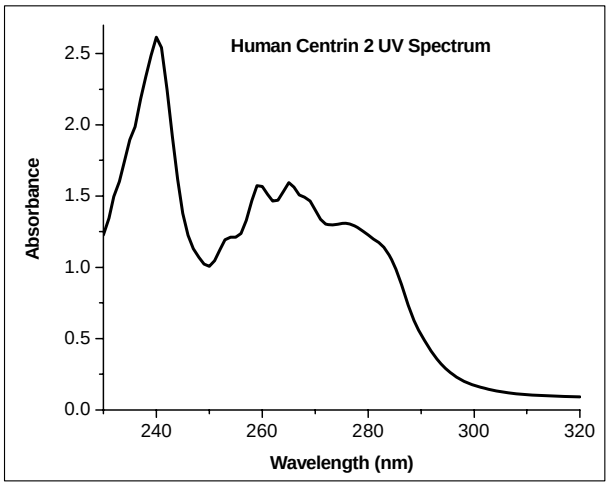


Figure 4.14: Characteristic *Human* centrin 2 UV spectrum. Human Centrin 2 UV spectrum showing characteristic absorbance of phenylalanine at 257.4 nm and tyrosine at 274.6 nm. The absence of absorbance of tryptophane at 280 nm means that there are no contaminatinig proteins since centrin does not have any tryptophane residue.

MASNFKKANM ASSSQRKRMS PKPELTEEQK QEIREAFDLF DADGTGTIDV KELKVAMRAL GFEPKKEEIK KMISEI DKEG TGKMNFGDFL TVMTQKMSEK DTKEEILKAF KLFDDDETGK ISFKN LKRVA KELGENLTDE ELQEMIDEAD RDGDGEVSEQ EFLRIMKKTS LY

Figure 4.15: Amino acid sequence of Hcen 2 cleavage products. The four EF-Hand motiff are boxed and the eleven amino terminal residues lost are shown in red and underline.

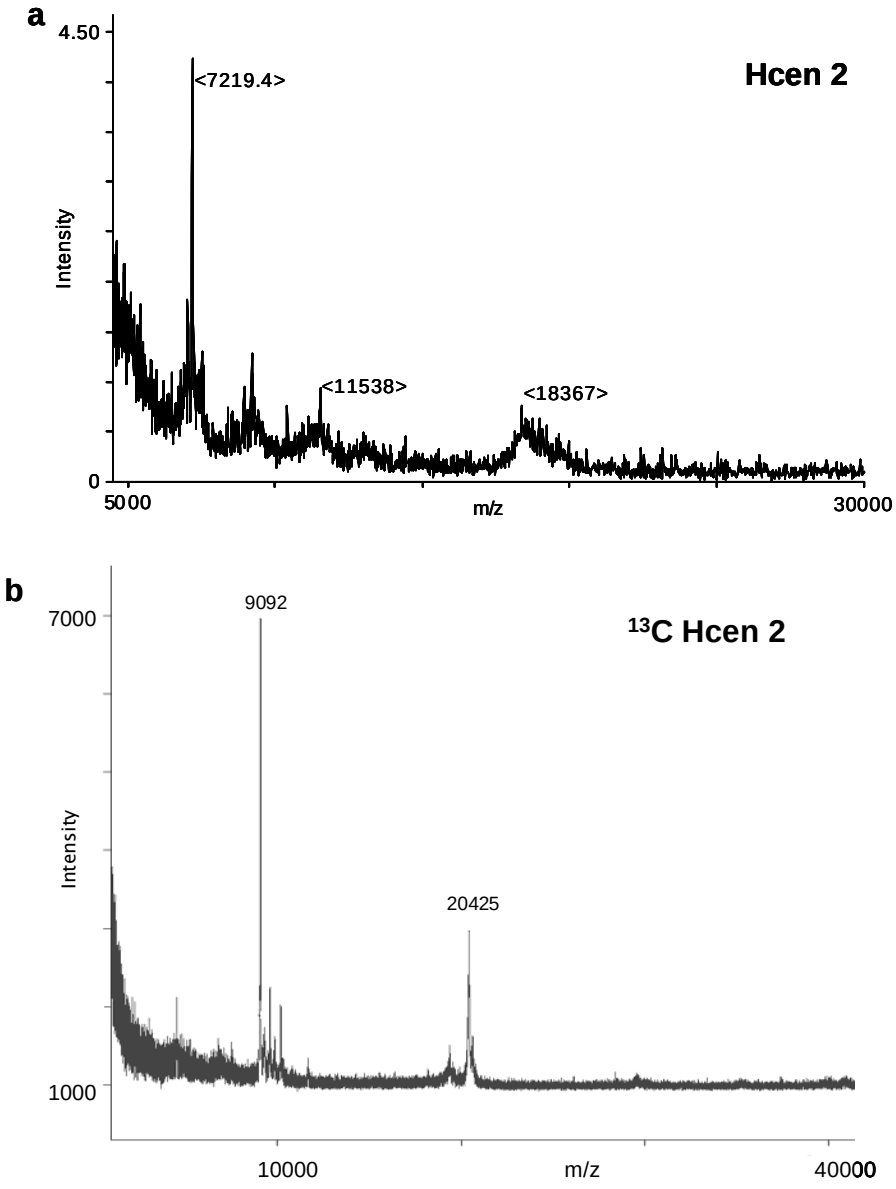


Figure 4.16: MALDI-TOF mass spectrometry results of Hcen 2 (a) **and ^{13}C Hcen 2** (b).

4.6 Differential Scanning Calorimetry (DSC)

In order to study the thermal unfolding process of Hcen 2, several DSC experiments were carried out varying the protein concentration and some VP-DSC setting parameters such as: scan rate (temperature increment per minute), filtering period (the time period over which data samples are averaged and a data point is generated and stored) and Feedback Mode/Gain (method and magnitude of cell-cell compensation used for thermal equilibration of the reference and sample cells), while all the experiments were carried out under the same buffer conditions (50 mM Hepes, 150 mM NaCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, pH 7.4). All samples were scanned within the temperature range of 10-127 ^{o}C.

Increasing the protein concentration resulted in a better definition of the transition temperature (established by determining peak maximum or minimum) as shown in Figure 4.17a and 4.17c, when a low (0.047 mM) protein concentration was used no discernable transition peak was observed, only a slight shoulder could be seen with a broad deflection showing an apparent pre-transition at ~86 ^{o}C. In contrast, when the protein concentration was increased (0.18 and 0.25 mM) a clear transition peak could be observed, but the peak became strongly negative, suggesting protein aggregation had occurred.

Alternatively when the protein concentration was kept at the same low range (0.042 vs. 0.047 mM) and variation were done in the Feedback mode/Gain from none (there is no active cell-cell compensation in response to temperature differences between the reference and sample cells) to high (provide for the greatest amount of cell-cell

compensation for any given temperature difference between the cells) there was a slight decline in the curve, now the shoulder becoming a peak with a more clear aggregation at ~ 120 ^{o}C (Figure 4.17a vs. 4.17b) and the pre-transition was constant at ~85 ^{o}C. Finally, when the protein concentration was kept between 0.1-0.2 mM and the Feedback mode/Gain was high a pretty well defined peak was observed; changes in filtering period from 8 to 16 did not result in peak changes. There were some variations in the pre-transition temperature and Tm between all runs, but the most repetitive Tm was near 120 ^{o}C (*data not shown*).

Human centrin 2 undergo a temperature-induced transition from the native to the unfolded state, with a pre-transition close to 85 ^{o}C (81-91) and melting temperature (T_m) at 120 ^{o}C. A single broad peak in heat absorption curve was observed and a clear aggregation negative peak was observed. Besides, the thermal denaturation of this protein was completely irreversible, as indicated by the absence of any endotherm in a second scan reheating the sample after slowly cooling to 10 ^{o}C (29) (Figure 4.18).

On the other hand turbidity was observed in samples that were heated up to 100 ^{o}C (the temperature at which the transition has been completed), indicating again that Hcen 2 is precipitated when heated at temperatures higher than 100 ^{o}C. In general, irreversible behavior during thermal unfolding studies is very common for proteins. Additionally, the absence of a post-transition baseline is due to the current technology which is limited to a maximum temperature of 130 ^{o}C. The observed absence of a stable post-transition base line did not permit us to calculate additional thermodynamic

parameters such as the excess heat capacity (C_p, sample minus reference) and the calorimetric enthalpy (ΔH_{cal}).

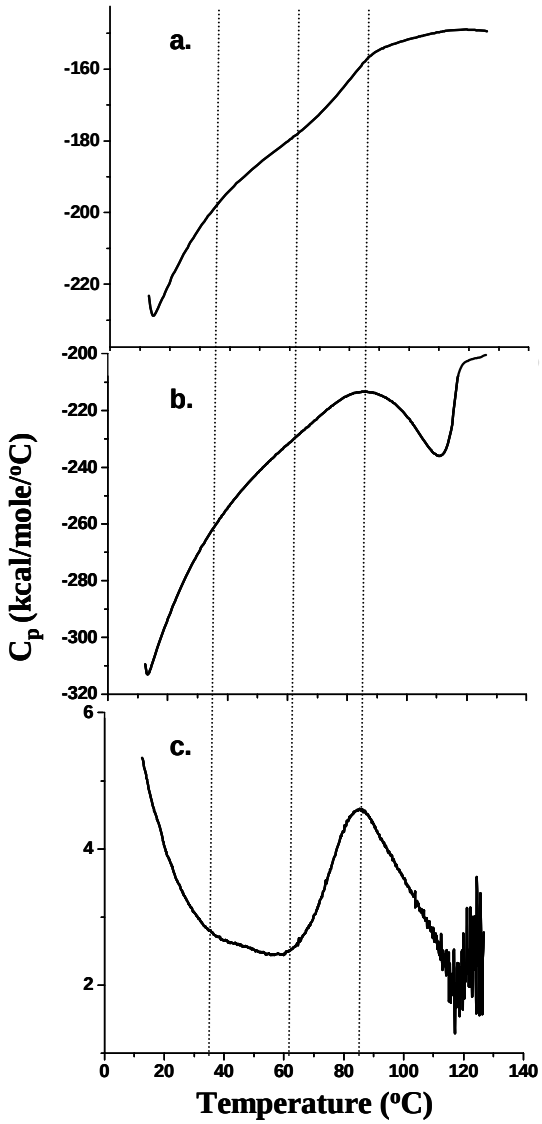


Figure 4.17: Human centrin 2 differential scanning calorimetry. This figure shows the effect of changes in protein concentration, scan rate and feedback mode/gain setting parameters in the definition of the thermal induce transition of Hcen 2. Scan a: protein concentration 0.047 mM (0.92 mg/dL), scan rate 90 ^{o}C/Hr and feedback mode/gain none; scan b: protein concentration 0.042 mM (0.84 mg/dL), scan rate 60 ^{o}C/Hr and feedback mode/gain high; scan c: protein concentration 0.25 mM, scan rate 60 ^{o}C/Hr and feedback mode/gain high.

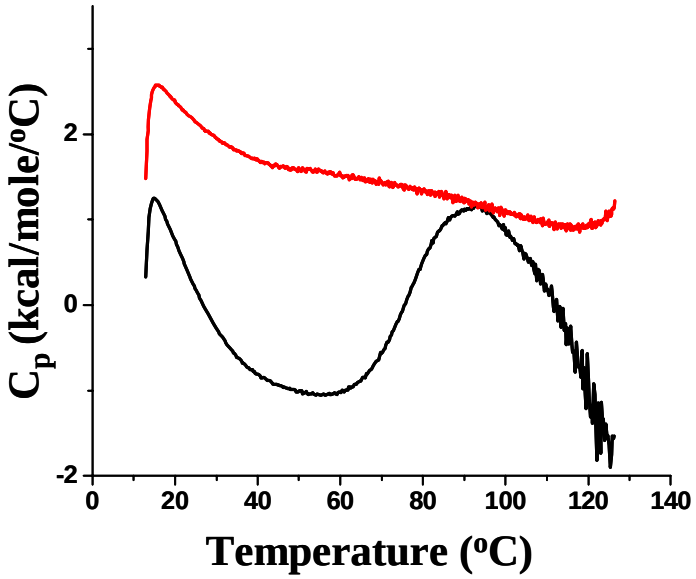


Figure 4.18: Irreversible Thermal Induce Denaturation of Hcen2. In black is shown the first Human centrin 2 (0.18 mM) scan in protein buffer (50 mM Hepes, 150 mM NaCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, pH 7.4); in red is shown the second scan (reheating) of the same sample with only an aggregation pattern and the absence of any endotherm.

5 Discussion and Conclusion

The high level expression in *E. coli*, purification and partial thermal characterization of recombinant *Human* centrin 2 has been described here. The strategy presented here for the purification of Hcen 2 is based on published protocols by Pastrana-Ríos et all (4) and Baron et all (25); however, it was optimized based on the known biochemical characteristic of the protein to achieve purification of centrin in the simplest and faster way and the most important consideration was to conserve the functionality of the protein.

The over expression induction process was tightly monitored by the use of a bioreactor controlling the environmental condition to ensure not only a maximum growth yield (pellet weight in grams), but also a maximum protein expression. Several over expression experiment were carried out, but the two major conclusion were that the use of a fresh plated colony to growth the overnight starting inoculum provide the best yield and that only one IPTG addition point was needed, since IPTG is not enzimatically degradated by bacterias *lac* operon. Even though variation of the addition point during different bacterial catabolic state were not tested; previous reports of recombinant protein over express induction protocols (35 and 36) show that best results were achieved when IPTG was added during early logarithmic phase.

Lee et all (35) also studied the acetate accumulation in glucose containing medium after induction as compared with that of glycerol containing medium, and

reported that the later resulted is less acetate accumulation, growth cessation and a higher productivity; future experiments must be carried out to resolve this issue.

The use of *E. coli* BL21-Gold (DE3) as host cells permit us to achieve an easy transformation process, this strain is generally good for protein expression due to its deficiency in *low* protease as well as the *ompT* outer membrane protease that can degrade proteins during purification (37). Even though of this feature and despite of the use of a protease inhibitor cocktail (2.0 mg/ml aprotinin, 0.5 mg/ml leupeptin, 1.0 mg/ml pepstatin A**)** we observed spontaneous cleavage of the first 11 to 12 amino acids.

A bioinformatic search was done to identify any potential protein kinase(s) or chemicals responsible for this behavior using the peptide cutter tool in the expert protein analysis system of the Swiss-protein database (**www.expasy.org**). This search suggest proteinase K as a possible candidate to cleavage at the eleventh peptide bond, which was the constant cleavage pattern observed (see appendix B) alternatively LysC, Thermolysin and Trypsin as possible candidates for cleavage at seventy first and eighty third peptide bonds, which were observed on just one protein batch, but we dischard Trypsin as a candidate because this enzyme is inhibit by two of the protease inhibitor used (aprotinin and leupeptin).

Here we have purified a 18,367 daltons native protein lacking its first amino terminal residues, but considered to remain functional because the four EF-hand canonical motif are preserved in their original position, more over there have been

reported some comparison with the closely related *Chlamydomonas* centrin (71% sequence identity) showing that the C-terminal domains of the two proteins have similar calcium binding properties, with two sites of significantly different affinities (in the μM and mM ranges) (8, 9 and 38). In contrast, the N-terminal domains exhibit a distinct behavior: the binding affinity of the *Chlamydomonas* domain is similar to that observed for Ca^{2+} sensor, while the Hcen 2 appears to be insensitive to divalent cations (8 and 38). This means that Hcen 2 C-terminal domain is responsible for its functional properties.

The spontaneous cleavage phenomenon of centrin during its purification process have been previously reported by Weber et all in 1994 (37) and more specifically for Hcen 2 by Durussel et all in 2000 (8) and Matei et all in 2003 (9) and more recently the same phenomenon have been described for Hcen 3 by Cox et all in 2005 (39). Here we propose the addition of a new protease inhibitor Pefabloc SC to prevent action of the possible trouble caused by Proteinase K. Proteinase K also known as endopeptidase K preferentially cleaves at aliphatic of aromatic amino acid residues in position P1. Ala in position P2 enhances the cleavage. The specificity of proteinase K is not always unambiguous and belongs to the peptidase family S8 (14).

We conclude that the purification protein presented here is suitable for large scale expression and purification of recombinant Hcen 2, while eliminating some bothersome processes like temperature precipitation, followed by several chromatographic steps (10) or the more sophisticated and expensive high pressure liquid chromatography (25) previously used.

DSC studies of native Hcen 2 show a thermal induced aggregation and denaturation at 120 ^{o}C with a T medium suggesting a possible pre-transition at 85 ^{o}C, this results are compatible with that observed for *Chlamydomonas* centrin where two pre-transitions were observed for the phosphorylated form of the protein, one at 20^{o}C and the second one at 70^{o}C, while for the unphosphorylated Ccen a 37^{o}C pre-transition was observed; indicating a more stable conformation for the unphosphorylated centrin. As obtained by DSC the Tm was determined to be 110ºC and 118ºC, for the phosphorylated and unphosphorylated forms of the protein, respectively (*manuscript in preparation*.).

6 Future Work

- Thermal dependence studies using infrared spectroscopy should be used to understand the molecular events that occur in this protein during its pre-transition (40-80 ^{o}C).
- To study the protein in its phosphorylated form using the DSC and infrared spectroscopy to establish its relative stability.
- ^{13}C-labeled protein should be used to study protein and target -peptide interactions.

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Appendix A

Nucleotide-Nucleotide Comparison between *Hcetn2* cDNA Insert and Available Hcen 2 Coding Sequence Reports using BLAST

The *Hcetn2* cDNA insert of pTP-6 molecule used in this research was compare to publish Human centrin 2 coding sequence found in *Homo sapiens* using nucleotide-nucleotide BLAST (Basic Local Alignment Search Tool) from the National Center for Biotechnology Information (NCBI) database (23). This comparison was done by inserting the *Hcetn2* cDNA sequence report shown in Figure 4.1, then choosing non-redundant sequence (nr) database and limiting the alignment to comparison with the first human centrin 2 nucleotide sequence reported by Lee and Huang in 1993 (12), with an entrez query X72964. The chosen alignment view was pairwise.

gi|441311|emb|X72964.1|HSCALT H.sapiens mRNA for caltractin
Length=1087

Score = 1005 bits (507), Expect = 0.0
Identities = 516/519 (99%), Gaps = 0/519 (0%)
Strand=Plus/Plus

```
Query  1
ATGGCCTCCAACTTTAAGAAGGCAAACATGGCATCAAGTTCTCAGCGAAAAAGAATGAGC  60

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  48
ATGGCCTCCAACTTTAAGAAGGCAAACATGGCATCAAGTTCTCAGCGAAAAAGAATGAGC  107

Query  61
CCTAAGCCTGAGCTTACTGAAGAGCAAAAGCAGGAGATCCGGGAAGCTTTTGATCTTTTC  120

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  108
CCTAAGCCTGAGCTTACTGAAGAGCAAAAGCAGGAGATCCGGGAAGCTTTTGATCTTTTC  167



Query  121
GATGCGGATGGAACTGGCACCATAGATGTTAAAGAACTGAAGGTGGCAATGAGGGCCCTG  180

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  168
GATGCGGATGGAACTGGCACCATAGATGTTAAAGAACTGAAGGTGGCAATGAGGGCCCTG  227

Query  181
GGCTTTGAACCCAAGAAAGAAGAAATTAAGAAAATGATAAGTGAAATTGATAAGGAAGGG  240

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  228
GGCTTTGAACCCAAGAAAGAAGAAATTAAGAAAATGATAAGTGAAATTGATAAGGAAGGG  287

Query  241
ACAGGAAAAATGAACTTTGGTGACTTTTTAACTGTGATGACCCAGAAAATGTCTGAGAAA  300

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  288
ACAGGAAAAATGAACTTTGGTGACTTTTTAACTGTGATGACCCAGAAAATGTCTGAGAAA  347

Query  301
GATACTAAAGAAGAAATCCTGAAAGCTTTCAAGCTCTTTGATGATGATGAAACTGGGAAG  360

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  348
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Query  361
ATTTCGTTCAAAAATCTGAAACGCGTGGCCAAGGAGTTGGGTGAGAACCTCGATGATGAG  420
```

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|||||||
Sbjct  408
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Query  421
GAGCTGCAGGAAATGATTGATGAAGCTGATCGAGATGGAGATGGAGAGGTCAGTGAGCAA  480

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  468
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Query  481  GAGTTCCTGCGCATCATGAAAAAGACCAGCCTCTATTAA  519
            ||||||||||||||||||||||||||||||||||||||
Sbjct  528  GAGTTCCTGCGCATCATGAAAAAGACCAGCCTCTATTAA  566


 Score = 24.3 bits (12),  Expect = 0.027
 Identities = 12/12 (100%), Gaps = 0/12 (0%)
 Strand=Plus/Minus

Query  320  TGAAAGCTTTCA  331
            ||||||||||||
Sbjct  378  TGAAAGCTTTCA  367
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Appendix B

Peptide Cutter Analysis of Hcen 2

7 PeptideCutter

3.2 You have selected the protein CETN2_HUMAN (P41208) from UniProtKB/Swiss-Prot :

Centrin-2 (Caltractin isoform 1).

7.1..1 The sequence to investigate:

```
1    MASNFKKANM ASSSQRKRMS PKPELTEEQK QEIREAFDLF DADGTGTIDV KELKVAMRAL
61   GFEPKKEEIK KMISEIDKEG TGKMNFGDFL TVMTQKMSEK DTKEEILKAF KLFDDDETGK
121  ISFKNLKRVA KELGENLTDE ELQEMIDEAD RDGDGEVSEQ EFLRIMKKTS LY
```

The sequence is 172 amino acids long.

7.1..2 Available enzymes

7.1..3 The enzyme(s) that you have chosen:

- Arg-C proteinase
- Asp-N endopeptidase
- BNPS_Skatole
- Caspase1
- Caspase2
- Caspase3
- Caspase4
- Caspase5
- Caspase6
- Caspase7
- Caspase8
- Caspase9
- Caspase10
- Chymotrypsin-high specificity (C-term to [FYW], not before P)
- Chymotrypsin-low specificity (C-term to [FYWML], not before P)
- Clostripain
- CNBr
- Enterokinase
- GranzymeB
- Factor Xa
- Formic acid
- Glutamyl endopeptidase
- Hydroxylamine
- Iodosobenzoic acid
- LysC
- NTCB (2-nitro-5-thiocyanobenzoic acid)
- Pepsin (pH1.3)
- Pepsin (pH>2)
- Proline-endopeptidase

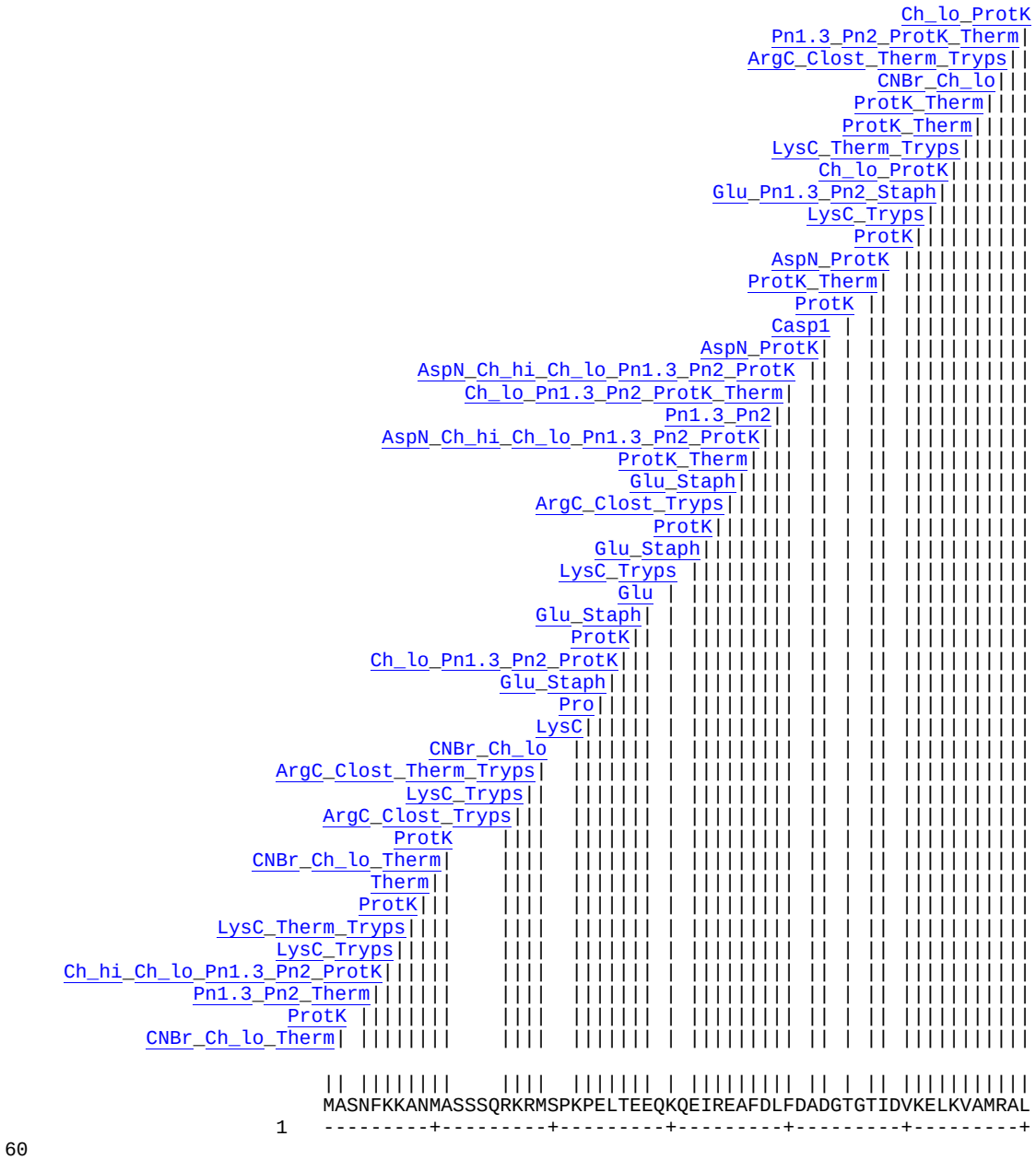
- Proteinase K
- Staphylococcal peptidase I
- Thermolysin
- Thrombin
- Trypsin

You have chosen to display all possible cleaving enzymes.

7.1..4

7.1..5 These are the cleavage sites of the chosen enzymes and chemicals mapped onto the entered protein sequence:

- You have chosen a block size of **60** for the map.
- Please note that the cleavage occurs at the **right side** (C-terminal direction) of the marked amino acid.
- You have the possibility ot display the results of a single enzyme by **mouseclicking** on the respective enzyme name in the map.


Ch_lo_ProtK
Pn1.3_Pn2_ProtK_Therm
ArgC_Clost_Therm_Tryps
CNBr_Ch_lo
ProtK_Therm
ProtK_Therm
LysC_Therm_Tryps
Ch_lo_ProtK
Glu_Pn1.3_Pn2_Staph
LysC_Tryps
ProtK
AspN_ProtK
ProtK_Therm
ProtK
Casp1
AspN_ProtK
AspN_Ch_hi_Ch_lo_Pn1.3_Pn2_ProtK
Ch_lo_Pn1.3_Pn2_ProtK_Therm
Pn1.3_Pn2
AspN_Ch_hi_Ch_lo_Pn1.3_Pn2_ProtK
ProtK_Therm
Glu_Staph
ArgC_Clost_Tryps
ProtK
Glu_Staph
LysC_Tryps
Glu
Glu_Staph
ProtK
Ch_lo_Pn1.3_Pn2_ProtK
Glu_Staph
Pro
LysC
CNBr_Ch_lo
ArgC_Clost_Therm_Tryps
LysC_Tryps
ArgC_Clost_Tryps
ProtK
CNBr_Ch_lo_Therm
Therm
ProtK
LysC_Therm_Tryps
LysC_Tryps
Ch_hi_Ch_lo_Pn1.3_Pn2_ProtK
Pn1.3_Pn2_Therm
ProtK
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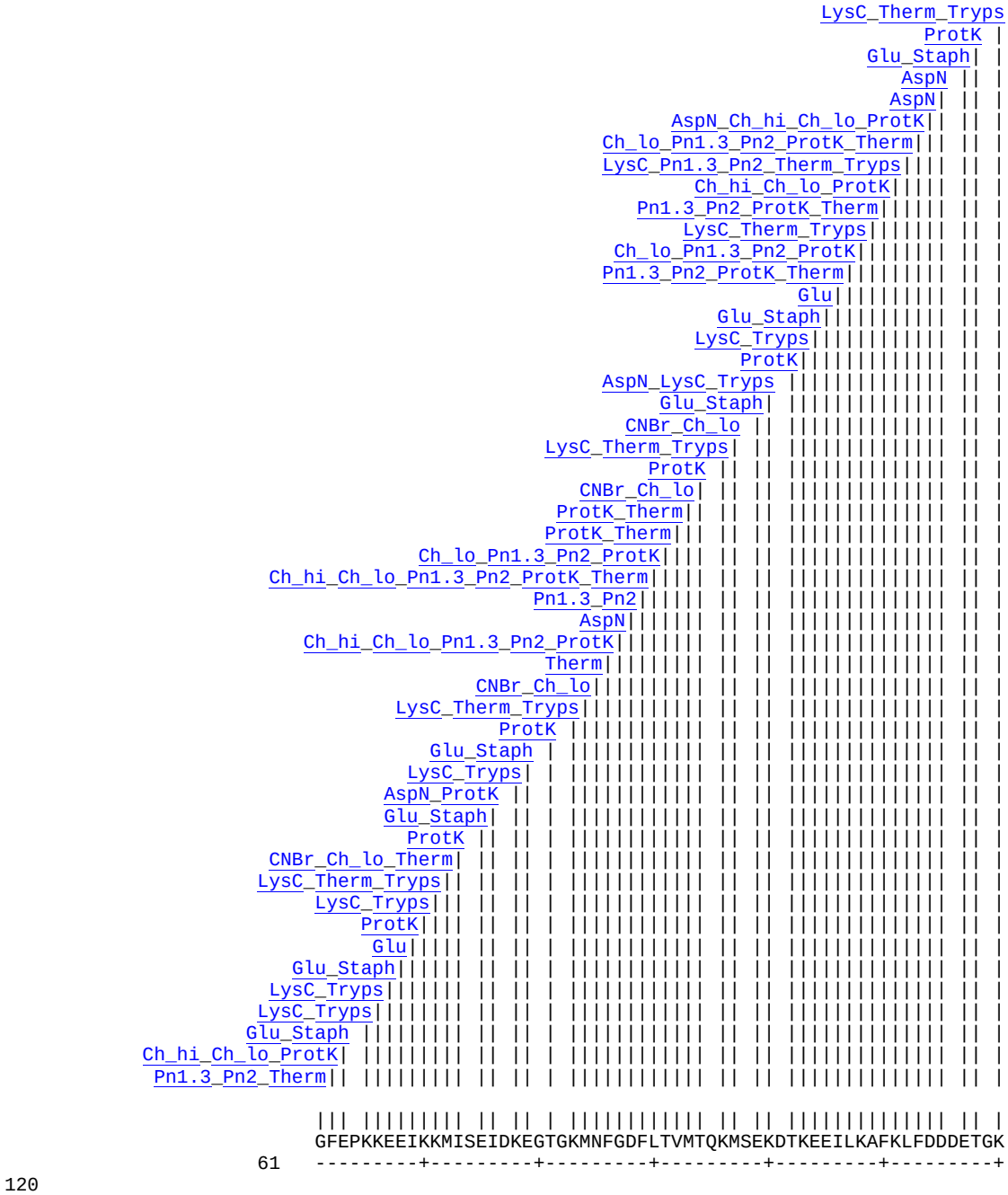
1
60


LysC_Therm_Tryps
ProtK
Glu_Staph
AspN
AspN
AspN_Ch_hi_Ch_lo_ProtK
Ch_lo_Pn1.3_Pn2_ProtK_Therm
LysC_Pn1.3_Pn2_Therm_Tryps
Ch_hi_Ch_lo_ProtK
Pn1.3_Pn2_ProtK_Therm
LysC_Therm_Tryps
Ch_lo_Pn1.3_Pn2_ProtK
Pn1.3_Pn2_ProtK_Therm
Glu
Glu_Staph
LysC_Tryps
ProtK
AspN_LysC_Tryps
Glu_Staph
CNBr_Ch_lo
LysC_Therm_Tryps
ProtK
CNBr_Ch_lo
ProtK_Therm
ProtK_Therm
Ch_lo_Pn1.3_Pn2_ProtK
Ch_hi_Ch_lo_Pn1.3_Pn2_ProtK_Therm
Pn1.3_Pn2
AspN
Ch_hi_Ch_lo_Pn1.3_Pn2_ProtK
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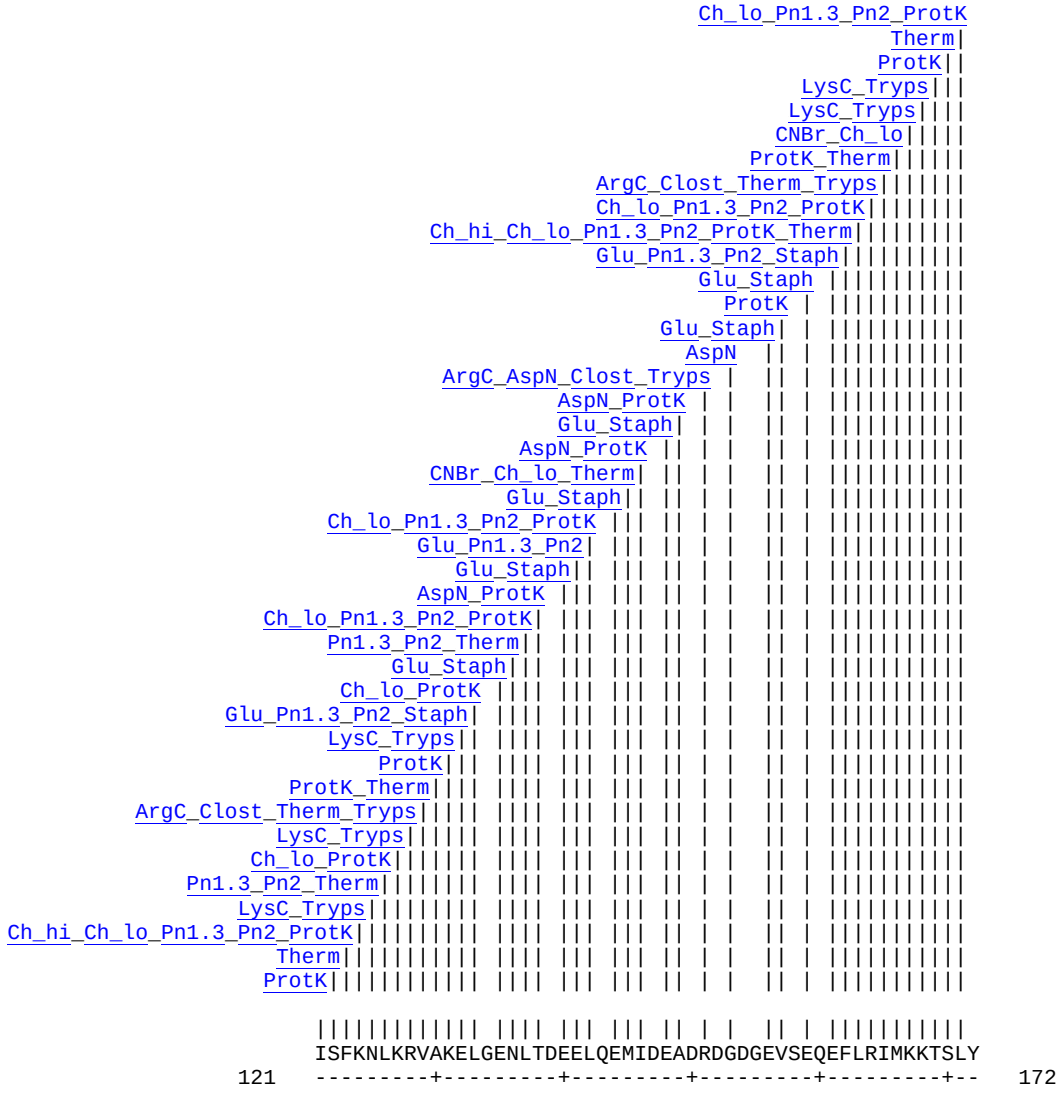
CNBr_Ch_lo
LysC_Therm_Tryps
ProtK
Glu_Staph
LysC_Tryps
AspN_ProtK
Glu_Staph
ProtK
CNBr_Ch_lo_Therm
LysC_Therm_Tryps
LysC_Tryps
ProtK
Glu
Glu_Staph
LysC_Tryps
LysC_Tryps
Glu_Staph
Ch_hi_Ch_lo_ProtK
Pn1.3_Pn2_Therm
GFEPKKEEIKKMISEIDKEGTGKMNFGDFLTVMTQKMSEKDTKEEILKAFKLFDDDETGK
61
120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Therm
ProtK
LysC_Tryps
LysC_Tryps
CNBr_Ch_lo
ProtK_Therm
ArgC_Clost_Therm_Tryps
Ch_lo_Pn1.3_Pn2_ProtK
Ch_hi_Ch_lo_Pn1.3_Pn2_ProtK_Therm
Glu_Pn1.3_Pn2_Staph
Glu_Staph
ProtK
Glu_Staph
AspN
ArgC_AspN_Clost_Tryps
AspN_ProtK
Glu_Staph
AspN_ProtK
CNBr_Ch_lo_Therm
Glu_Staph
Ch_lo_Pn1.3_Pn2_ProtK
Glu_Pn1.3_Pn2
Glu_Staph
AspN_ProtK
Ch_lo_Pn1.3_Pn2_ProtK
Pn1.3_Pn2_Therm
Glu_Staph
Ch_lo_ProtK
Glu_Pn1.3_Pn2_Staph
LysC_Tryps
ProtK
ProtK_Therm
ArgC_Clost_Therm_Tryps
LysC_Tryps
Ch_lo_ProtK
Pn1.3_Pn2_Therm
LysC_Tryps
Ch_hi_Ch_lo_Pn1.3_Pn2_ProtK
Therm
ProtK
ISFKNLKRVAKELGENLTDEELQEMIDEADRDGDGEVSEQEFLRIMKKTSLY
121
172

Appendix C

Targeted Mutations In Centrin Phosphorylation Motif Using a Gene Expression System

The summarized work presented in this appendix was perfomed during my 2004 summer graduate visit to Dr, Jeffrey. L. Salisbury's Laboratory in the Biochemistry and Molecular Biology Department, at Mayo Clinic School of Medicine in Rochester, Minnesota. Dr. Salisbury has generously agreed with the inclusion of this research as an appendix in this thesis report. Also the data presented here includes only partial results.

Introduction

In 2001 Lutz et al (6) published the results of their research conducted to study cell cycle dependent phosphorylation of centrin. Using an antibody that was specific for phosphorylated centrin and standard biochemical methods, they demonstrated that in cultured vertebrate cells, centrin is phosphorylated near its carboxyl terminus at serine residue 170 early in mitosis when the newly duplicated centrosomes separate to give rise to mitotic spindle pole. The spindle pole localization of phosphocentrin remains high until metaphase and then diminishes to basal level by telophase (Figure 1).

They also reported that the timing of centrin phosphorylation suggests that this process may initiate the separation of duplicated centrosomes in preparation for mitotic spindle formation. Experimental elevation of protein kinase A (PKA) activity in interphase cells also resulted in the phosphorylation of centrin at serine residue 170 and the concomitant movement of the centrioles away from one another, in a manner similar

to the transient separation of the pair of centrioles that normally occurs preceding centrosome duplication which begins at about the time of the G_1/S transition (Figure 2).

In this study there was reported the identified (29, 30) target consensus motif for protein kinase A in the carboxy terminus of the three human isoforms, as well as in the *Xenopus* and *Chlamydomonas* centrin, see Table I.

The contribution made by Lutz et al resulted in a better unsderstanding of centrin phosphorylation during the cell cycle, but the functional consequences of centrin phosphorylation are still not known. The main objective of the project presented here was to elucidate the functional consequences of centrin phosphorylation during the cell cycle in HeLa cells. To accomplish this we targeted the following specific objective:

- Comparing the behavior of centrosome carrying either wild type centrin or centrin with mutated carboxy-terminus phosphorylation motif through a highly regulated gene expression system.

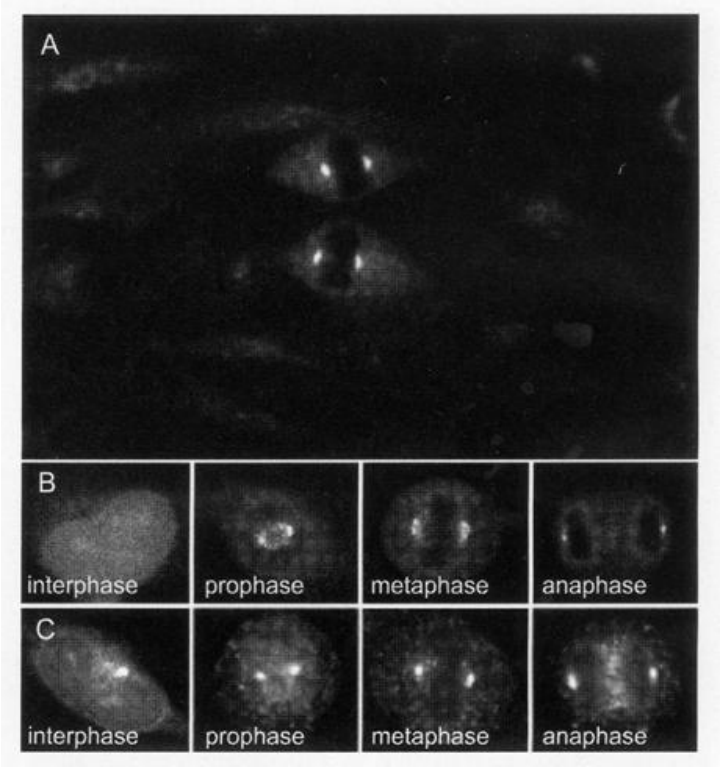

Figure 1: Phosphocentrin is localized at the spindle poles of HeLa cells. Indirect inmunofluorescence micrographs of HeLa cells stained with αHCT-P IgG (A & B) and 26/14-1 (C). A and B, αHCT-P IgG only stains cells in prophase and metaphase intensily with diminishing label in anaphase cells and only low levels of diffuse label in interphase cells. C, 26/14-1 labels centrin regardless of the stage of cell cycle.
Figure taken from Lutz et all. (2001) J. Biol. Chem. 276, 20774-20780.

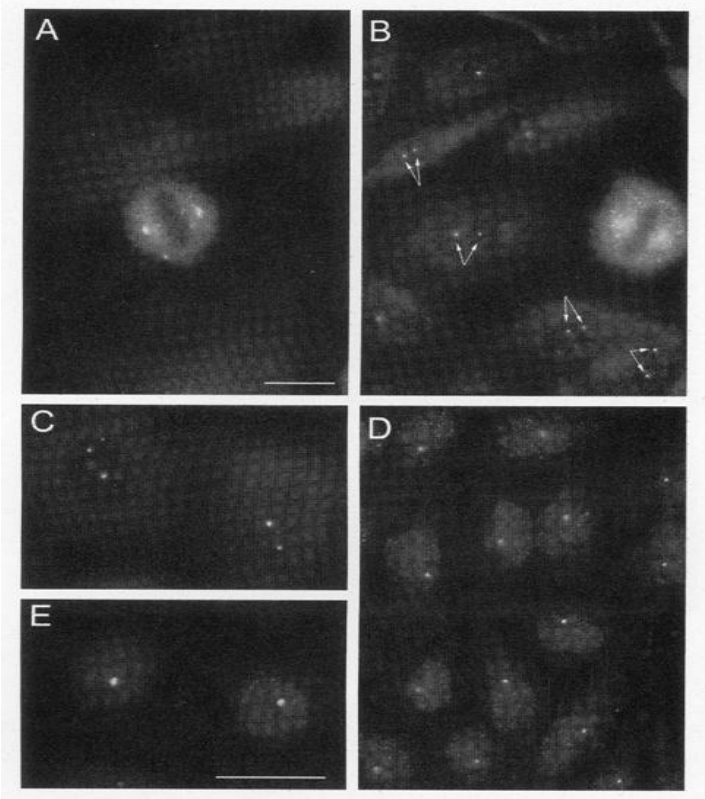

Figure 2: Stimulation of PKA results inextraordinary phosphorylation of centrosomes and in centriole separation in interphase HeLa cells. A, indirect inmunofluoresce of HeLa cells stained with αHCT-P IgG (against P-centrin) intensely stain metaphase spindle poles. B and C, after stimulation of PKA, αHCT-P IgG stain interphase centrosomes and mitotic spindle poles. D and E, control cells satined with 26/14-1 show typical of interphase centrosomes.
Figure taken from Lutz et all. (2001) J. Biol. Chem. 276, 20774-20780.

TABLE I
Carboxy-terminal amino acid sequences for centrin

Species	Accession No.	Sequence
A. Carboxy-terminal 12 amino acid residues of centrin		
Human 1	U03270	^{160}EEFLRIMKKTSLY^{172}
Human 2	X72964	^{160}QEFLRIMKKTSLY^{172}
Human 3	Y12473	^{158}EEFIAIMGDI^{167}
Xenopus	U37538	^{160}QEFLRIMKKTSLF^{172}
Chlamydomonas	X57973	^{157}DEFIRIMKKTSLF^{169}
B. PKA phosphorylation consensus motif		
		KK*X*S***X* consensus (26)
		KKTS*LY human CETN1 and CETN2
C. Synthetic peptides		
HCT-12		cEFLRIMKKTSLY
HCT-P		cEFLRIMKKTS^{P}LY

Taken from Lutz et all (2001) *J. Biol. Chem*. 276, 20774-20780.

Materials and Methods

Four different DNA constructions were done using the BD^{TM} Tet-On Gene Expression System and cDNA carrying either wild type Human centrin 2 whole sequence or several previously mutated cDNAs carrying whole Human centrin 2 sequence with targeted mutations in the carboxy-terminus phosphorylation consensus motif (this mutations were previously done by Dr. Salisbury group). The cDNA Hcetn 2 insert were place in the Cla I and Not I restriction sites of the pTRE2hyg2-HA (expression vector). The BD^{TM} Tet-On Gene Expression System was created to use in conjunction with the the BD^{TM} HeLa Tet-On cell lines to achieve a regulated high level gene expression, which is turned on by the addition of doxycycline (Dox) to the culture media.

Hela Tet-On cells were previously transfected by a liposome-mediated transfection technique in five different reaction shown in Table II with several clones for

each reaction; and grown in Dulbecco's Modified Eagle's Medium supplemented with fetal bovine serum (FBS), L-Glutamine, streptomycine sulfate, neomycin (selection for the regulatory plasmid) and hygromycin (selection for the response plasmid). When double stable transfected cell lines were achieve containing both the regulatory and response plasmids, this clones were cultured until they reach at least an 90% confluence, and then trypsinezed and split to prepare frozen stocks and induce gene expression.

Gene expression induction were assessed by duplicate culture of each clone one containing doxycycline and another without doxycycline, when confluent this cells were lysed followed by a single sonication pulse and total protein concentration was measured by DC protein assay. Final screen was done by western blot using four types of antibodies: one monoclonal antibody against the HA-tag (Santa Cruz), two polyclonal antibodies against the HA-tag (Clontech lot 97991 and lot 98056) and the monoclonal antibody anti- Centrin 20H5.

Gene expression system

This expression system is based on the principle that in *E. coli*, the Tet repressor protein (TetR) negatively regulates the genes of the tetracycline-resistance operon on the Tn 10 transposon. TetR blocks transcription of these genes by binding to the tet operator sequence (tetO) in the absence of tetracycline (Tc) (31).

The first critical component of the Tet system is the regulatory protein, based on TetR. In the Tet-Off System described by Gossen and Bujard in 1992 (32), this 37-kDa

protein is a fusion of amino acids 1-207 of the TetR and the C-terminal 127 amino acids of the Herpes simplex virus VP16 activation domain, this addition converts the TetR from a transcriptional repressor to a transcriptional activator, known as tetracycline-controlled transactivator (tTA).

The Tet-On system is similar to the Tet-Off system, but the regulatory protein is based on a "reverse" Tet repressor (rTetR) which was created by four amino acids changes in TetR (33). The resulting protein, rtTA (reverse tTA), is encoded by the pTet-On regulator plasmid, which also includes a neomycin-resistence gene to permit selection of stably transfected cells.

The second critical component is the response plasmid which expresses a gene of interest (Hcetn2) under control of the tetracycline-response element, or TRE. pTRE2hyg2-HA is a Tet responsive vector that allowed us to express human centrin 2 bearing a hemagglutinin tag (HA). This vector contains an MCS (multiple cloning site) immediately downstream of a sequence that codes for the HA tag. Transcription is regulated by the Tet-responsive promoter P_{hCMV}. This promoter contains the TRE, which consist of seven copies of the 19-bp tet operator sequence (tetO). The TRE element is just upstream of the minimal CMV promoter (P_{minCMV}), which lacks the enhancer that is part of the complete CMV promoter. Consequently, P_{hCMV} is silent in the absence of binding of rtTA to the tetO sequence (Figure 3). pTRE2hyg2-HA also contain the hygromycin resistence gene for direct selection of stable transformants.

Table II

Construction of Hcetn 2 Recombinant Clones

Construction Name	Vector	Insert
pJLS-212	pTREhyg2-HA	No (empty)
pJLS-206	pTREhyg2-HA	Wild type Centrin ^{167}KKTSLY^{172}
pJLS-200	pTREhyg2-HA	Hcen2 C-terminal mutation ^{167}KKTSGG^{172}
pJLS-204	pTREhyg2-HA	Hcen2 C-terminal mutation ^{167}KKAALY^{172}
pJLS-205	pTREhyg2-HA	Truncated C-terminal Hcen2

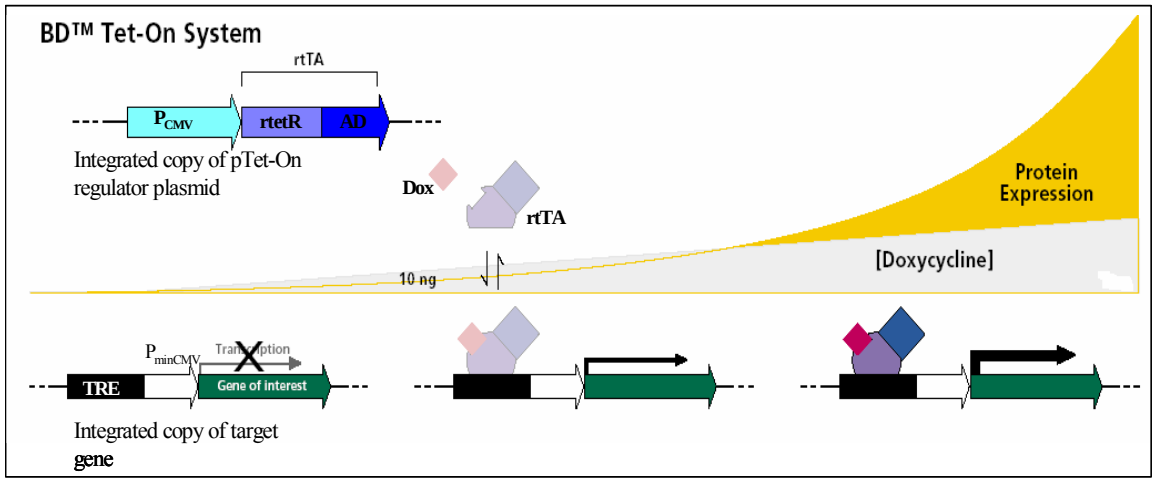

Figure 3: BD Tet-On System. Taken from Tet system user manual (BD Biosciences Clontech). http://www.clontech.com/clontech/products/literature/pdf/brochures/TetBR.pdf.

Results and Conclusions

- The monoclonal HA-tag antibody recognizes control GFP-HA Calmodulin and HA-Centrin expressed in HeLa Tet-on clones by western blot and produces less back ground than the polyclonal HA-tag antibody.

- Using this method, we were able to identify 3 p200 positive clones and 1 p204 positive clone.

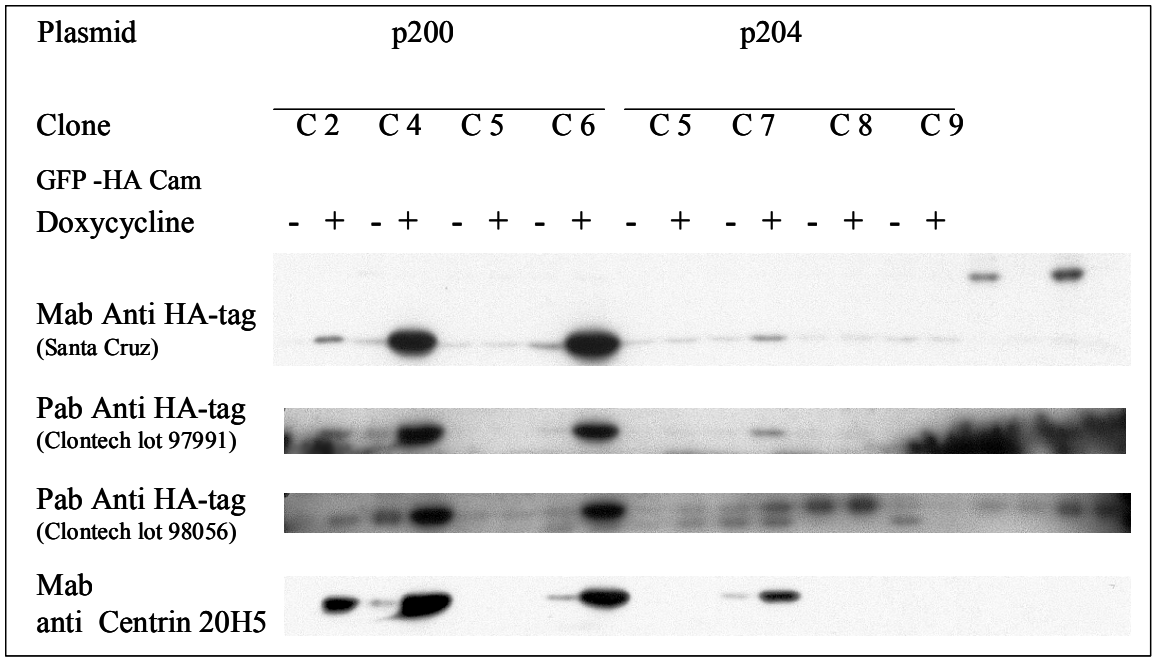

Figure 4: Western-blot Screening results of p200 and p204 HeLa Tet-On Stable Transformants.

Future Work

- Screen all the remaining clones by western blot and immunofluorescence.
- Analyze the *in vivo* behavior of each Centrin mutation during the cell cycle in the Hela Tet-on system.
- Construct a new plasmid where the 167KK168 are mutated.
- Site-directed mutagenesis of other potential phosphorylation sites on Centrin.