

**A CTG Polymorphism in the *CNDP1* gene determines the secretion of serum carnosinase in Cos-7 transfected cells.**

Received for publication 5 February 2007 and accepted in revised form 15 June 2007.

Riedl E<sup>1\*</sup> and Koeppel H<sup>1\*</sup>, Brinkkoetter P<sup>1</sup>, Sternik P<sup>1</sup>, Steinbeisser H<sup>2</sup>, Sauerhoefer S<sup>1</sup>, Janssen B<sup>2</sup>, van der Woude FJ<sup>1†</sup>, Yard BA<sup>1</sup>

\* Equally contributing authors

From the departments of:

<sup>1</sup> Department of Nephrology, Endocrinology and Rheumatology, Fifth Medical Clinic, Mannheim, Germany

<sup>2</sup> Institute of Human Genetics Heidelberg, Heidelberg, Germany

† deceased

Correspondence to:

Hannes Koeppel, V. Med. Klinik, Universitätsklinikum Mannheim, Theodor-Kutzer-Ufer 1-3, D-68167 Mannheim, Germany

e-mail: hannes.koeppel@med5.ma.uni-heidelberg.de

## **ABSTRACT**

Recently, we demonstrated that a polymorphism in exon 2 of the serum carnosinase (CNDP1) gene is associated with susceptibility to develop diabetic nephropathy. Based on the number of CTG repeats in the signal peptide, 5 different alleles coding for 4, 5, 6, 7 or 8 leucines (4L to 8L) are known. Diabetic patients without nephropathy are homozygous for the 5L allele more frequently than those with nephropathy. Since serum carnosinase activity correlates with *CNDP1* genotype, we hypothesized in the present study that secretion of serum carnosinase is determined by the *CNDP1* genotype. To test this hypothesis, we transfected COS-7 cells with different *CNDP1* constructs varying in CTG repeats and assessed the expression of CNDP1 protein in cell extracts and supernatants. Our results demonstrate that CNDP1 secretion is significantly higher in cells expressing variants with more than 5 leucines in the signal peptide. Hence, our data might explain why individuals homozygous for 5L have low serum carnosinase activity. Because carnosine, the natural substrate for carnosinase, exerts anti-oxidative effects, inhibits ACE activity and AGE formation, our results support the finding that diabetic patients homozygous for *CNDP1* 5L are protected against DN.

## INTRODUCTION

Diabetic nephropathy (DN) is the most frequent cause for end stage renal disease in the western world (1). The incidence of DN is approximately 40 percent in type 1 and type 2 diabetic patients (2). Although major risk factors for development and progression of diabetic nephropathy, e.g. poor glycaemic control and hypertension, have been identified, DN can still develop in diabetic patients with well controlled blood glucose concentrations (3). Also, in hypertensive diabetic patients the speed of renal function deterioration can be reduced by appropriate treatment, but treatment as such does not eliminate the susceptibility to develop DN (4).

A number of epidemiologic studies have suggested that susceptibility to develop DN is genetically determined (5, 6). In a linkage analysis performed on 18 Turkish families with type 2 diabetes and nephropathy, we have previously identified a susceptibility locus for DN on chromosome 18q22.3-q23. Association between DN and this locus was subsequently confirmed in Pima Indians (7) and Afro-Americans (8). Recently, we have narrowed down this locus and found that susceptibility to develop DN was related to the presence of a polymorphism in a single gene, the *CNDP1* gene (9), encoding the serum carnosinase protein.

Serum carnosinase, a dipeptidase belonging to the M20 metalloprotease family, is the rate limiting enzyme for the hydrolysis of carnosine into  $\beta$ -alanine and histidine. Carnosine is an antioxidant (10), inhibits nonenzymatic glycosylation (11), prevents high glucose induced extracellular matrix accumulation (9) and prevents crosslinking of proteins caused by reactive aldehydes (11). In light of these biochemical properties, it becomes clear that carnosine might be a modulator of hyperglycaemia induced damage. Moreover, since carnosine is also a natural ACE inhibitor (12) and pharmacologic

inhibition of the renin-angiotensin-system is known to decline progression of DN (13, 14), carnosine can be considered as a protective factor for DN. It must be stressed however that the findings related to carnosine as ACE inhibitor must be interpreted with some precaution as the concentration of carnosine to inhibit ACE is approximately 5.26 mM and thus not in a physiological range (21). Furthermore, although serum carnosine concentrations rapidly increase after ingestion of meat, in general serum carnosine concentrations are very low and decrease after meat ingestion within 5 hours to almost undetectable levels (22).

Similar to all secreted proteins, *CNDP1* is synthesized as a precursor containing an N-terminal signal peptide sequence. This enables the nascent protein to be targeted to the endoplasmic reticulum. Signal peptides contain in general a hydrophobic stretch flanked by polar N-terminal and C-terminal domains (15). The hydrophobic domain is of utmost importance for the function of the signal peptide (16), i.e. targeting the protein into the secretory pathway. There is a (CTG)<sub>n</sub> polymorphism (D18S880) located within the signal peptide of the human serum-carnosinase. This results in a signal peptide containing a hydrophobic stretch of 4, 5, 6, 7 or 8 leucines (4L, 5L, 6L, 7L and 8L alleles). Since diabetic patients homozygous for the 5L allele of *CNDP1* are protected for DN and serum carnosinase activity is low in these patients, we tested whether carnosinase secretion is determined by the *CNDP1* genotype. Another polymorphism within the *CNDP1* signal peptide is the arginine to glycine transition in codon 6 (R6G; c.16G>A; rs11151964). The secretion efficiency of this variant, which has not been mentioned in relation to DN before, was investigated in the present study as well.

## MATERIALS AND METHODS

### *Construction of the CNDPI gene variants*

A cDNA clone of the *CNDPI* gene (RZPD Library 983, entry No BX094414) containing 6 CTG-repeats was kindly provided by Dr. M. Moeller (Dept. of Nephrology, RWTH, Aachen, Germany) and used as template. In three sequential PCR steps, *CNDPI* variants containing 4, 5, 6, 7 or 8 CTG-repeats were constructed. PCR reactions were performed according to standard methods; sequences of the primers used in each step are depicted in table 1. The 6L variant was constructed with either adenine (A) or guanine (G) at position +16. All *CNDPI* constructs were cloned into the pCS II + mt vector to generate a myc-tagged carnosinase protein. Transformation was performed in competent *E.coli* (TOP10F', Invitrogen, Karlsruhe, Germany) using blue-white screening (Sigma, Steinheim, Germany).

### *Cell culture and transfection*

Cos-7 cells (Invitrogen, Karlsruhe, Germany) were transiently transfected using Nanofectin (PAA The Cell Culture Company, Pasching, Austria) as recommended by the manufacturer. Cell supernatants were collected and cell lysates were prepared 48 hours after transfection.

### *Western blot analysis*

Samples were denatured, separated by a 8% SDS-PAGE and transferred to a PVDF membrane (Roche, Mannheim, Germany) by means of semi-dry blotting. Three different antibodies were used for the detection of carnosinase: anti-carnosinase K18K polyclonal antibody (kindly supplied by M. Teufel, Sanofi Synthelabo Recherche, Strasbourg), anti-carnosinase polyclonal (R&D Systems, Wiesbaden-Nordenstadt Germany) or anti-myc (abcam, Cambridge, United Kingdom). After incubation with appropriate secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany) antibody binding was visualized by

chemiluminescence (PerkinElmer, Boston, USA). Intensity of the bands was measured by densitometry using the ImageJ 1.36b software. In some experiments aliquots of the protein samples were deglycosylated with PNGase F (New England Biolabs, Frankfurt, Germany) before SDS-PAGE.

### *Statistical analysis*

Statistical analysis was performed with SAS software (SAS release 8.02, SAS Institute Incorporated, Cary, USA). In order to investigate the influence of Leucine adjusted for time on the percentage of carnosinase, a two-way ANOVA has been used. Difference of secretion efficiency of  $\leq 5L$  to  $\geq 6L$  was shown by planned linear contrasts using the SAS procedure GLM together with CONTRAST statements. Test results with  $p < 0.05$  were considered to be significant.

## RESULTS

To test the hypothesis that the length of the hydrophobic leucine stretch in the signal peptide of serum carnosinase influences the efficiency of carnosinase secretion, we constructed myc-tagged *CNDPI* gene variants differing in the number of CTG repeats in the signal sequence. Constructs encoding 4, 5, 6, 7 or 8 leucines, representing all thus far known alleles for *CNDPI*, were cloned into Cos 7 cells.

In cell extracts of *CNDPI* transfected cells, immune-reactive bands were detected in Westernblot analysis with apparent molecular weight of 86 and 88 kDa respectively. In supernatants of the transfected cells, a single dominant band of approximately 92 kDa was present. Similar data were obtained when monoclonal anti-myc or polyclonal anti-carnosinase antibodies were applied to the Westernblot. In cells transfected with the empty vector, no immune-reactive bands were detected (Fig 1). After PNGase treatment of supernatant and cell extracts, a single band of approximately 86 kDa was found (Fig 2),

suggesting differences in N-glycosylation between both samples

The percentage of carnosinase secreted in the supernatants of all transfectants was assessed by densitometry (Fig 3A). In Cos-7 cells expressing CNDP1 containing a 4 or 5 leucine stretch, more carnosinase was found in the cell extracts than in the corresponding supernatants ( $\leq 5L$  vs.  $\geq 6L$   $p < 0.0001$ ). In contrast, Cos-7 cells expressing gene variants of CNDP1 with more CTG repeats clearly secreted carnosinase much better. With increasing length of the CTG repeat the percentage of secreted carnosinase increased (Fig 3B). In cells transfected with a CNDP1 construct lacking the complete signal peptide, no carnosinase could be detected in supernatants (Fig 3A).

To test if the A to G polymorphism at position +16 (R6G) was also relevant for carnosine secretion, CNDP1 6L constructs were generated with either adenine or guanine at position +16. No difference in secretion efficiency of carnosinase was found for these variants (6A=47.4±6.0 %, 6G=46.1±5.2 %,  $p=0.2707$ , data not shown).

## DISCUSSION

Recently, we have demonstrated that a polymorphism situated in the signal peptide of CNDP1 to be associated with susceptibility to develop DN in diabetic patients (9). Association of this polymorphism with DN has recently been confirmed in European Americans (17). Five different variants of the signal peptide, that all differ in the length of the hydrophobic leucine stretch, were identified in population screenings. A null-allele with an insertion of 5 nucleotides within the leucine repeat was also detected, but due to a frame shift this allele does not code for a proper carnosinase protein (18).

The importance of the (CTG)<sub>n</sub> repeat located in the hydrophobic core of the signal peptide is that it influences the efficiency of

carnosinase secretion, as demonstrated in the present study. While carnosinase encoded by CNDP1 containing the (CTG)<sub>4</sub> and (CTG)<sub>5</sub> repeat is poorly secreted, a more efficient secretion occurs when the CNDP1 variants contain more than 6 CTG repeats. Our results are in line with theoretical calculations based on the G. von Heijne scores. Moreover our data provide experimental evidence, explaining the association of the CNDP1 genotype and serum carnosinase activity (9).

In the signal peptide of CNDP1 there is an additional single nucleotide polymorphism (SNP) present resulting in either in either adenine or guanine at position +16 (R6G). Because the A/G SNP and (CTG)<sub>n</sub> repeat are not in disequilibrium all possible allelic combinations of A/G and (CTG)<sub>n</sub> do occur. We now also show that the A/G SNP does not influence carnosinase secretion efficiency.

Post- translational modification of serum carnosinase has previously been demonstrated and includes both N- and O-glycosylation (19). Three putative N-glycosylation sites are found in the carnosinase sequence. Secreted and non-secreted carnosinase differ in the extent of N-glycosylation as could be demonstrated by PNGase treatment. This therefore explains the difference in apparent molecular weight between both. We are aware that N-glycosylation can influence protein secretion (20). However, secreted carnosinase from all different CNDP1 variants were to a similar extent N-glycosylated as no difference in molecular weight was detected between the variants. The importance of N-glycosylation for secretion efficiency of carnosinase *per se* was not tested in this study, because genetic evidence for the association of serum carnosinase activity and N-glycosylation of carnosinase is lacking.

Serum carnosinase deficiency is associated with mental retardation and sensory peripheral neuropathy (23, 24). Also alcoholics with abnormal muscle biopsy findings have low

serum carnosinase activity (25), however, it is unclear if the (CTG)<sub>n</sub> polymorphism also plays a role in serum carnosinase activity in this group of individuals.

In conclusion we show that the (CTG)<sub>n</sub> polymorphism in the signal peptide of the *CNDP1* gene is functional, determining secretion efficiency of serum carnosinase. Our data also explains why serum carnosinase activity in individuals homozygous for *CNDP1* 5L is low, resulting in relatively high levels of the renoprotective dipeptide carnosine.

#### **ACKNOWLEDGEMENTS:**

This project was partially funded by the PREDICTIONS-Project, an EU-funded network of the 6<sup>th</sup> framework (LSHM-CT-2005-018733).

We thank Dr. C. Weiss, Institute of Medical Statistics of our Faculty, for the statistics calculations.

## References

1. Ritz, E., Rychlik, I., Locatelli, F., and Halimi, S.: End-stage renal failure in type 2 diabetes: A medical catastrophe of worldwide dimensions. *Am J Kidney Dis* 34:795-808, 1999
2. Hasslacher, C., Ritz, E., Wahl, P., and Michael, C.: Similar risks of nephropathy in patients with type I or type II diabetes mellitus. *Nephrol Dial Transplant* 4:859-863, 1989
3. UK Prospective Diabetes Study (UKPDS) Group: Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 352:837-853, 1998
4. Parving, H.H., Andersen, A.R., Smidt, U.M., and Svendsen, P.A.: Early aggressive antihypertensive treatment reduces rate of decline in kidney function in diabetic nephropathy. *Lancet* 1:1175-1179, 1983
5. Seaquist, E.R., Goetz, F.C., Rich, S., and Barbosa, J.: Familial clustering of diabetic kidney disease. Evidence for genetic susceptibility to diabetic nephropathy. *N Engl J Med* 320:1161-1165, 1989
6. Freedman, B.I., Bowden, D.W., Sale, M.M., Langefeld, C.D., and Rich, S.S.: Genetic susceptibility contributes to renal and cardiovascular complications of type 2 diabetes mellitus. *Hypertension* 48:8-13, 2006
7. Vardarli, I., Baier, L.J., Hanson, R.L., Akkoyun, I., Fischer, C., Rohmeiss, P., Basci, A., Bartram, C.R., Van Der Woude, F.J., and Janssen, B.: Gene for susceptibility to diabetic nephropathy in type 2 diabetes maps to 18q22.3-23. *Kidney Int* 62:2176-2183, 2002
8. Bowden, D.W., Colicigno, C.J., Langefeld, C.D., Sale, M.M., Williams, A., Anderson, P.J., Rich, S.S., and Freedman, B.I.: A genome scan for diabetic nephropathy in African Americans. *Kidney Int* 66:1517-1526, 2004
9. Janssen, B., Hohenadel, D., Brinkkoetter, P., Peters, V., Rind, N., Fischer, C., Rychlik, I., Cerna, M., Romzova, M., de Heer, E., et al.: Carnosine as a protective factor in diabetic nephropathy: association with a leucine repeat of the carnosinase gene CNDP1. *Diabetes* 54:2320-2327, 2005
10. Boldyrev, A., Bulygina, E., Leinsoo, T., Petrushanko, I., Tsubone, S., and Abe, H.: Protection of neuronal cells against reactive oxygen species by carnosine and related compounds. *Comp Biochem Physiol B Biochem Mol Biol* 137:81-88: 2004
11. Hipkiss, A.R., Preston, J.E., Himsworth, D.T., Worthington, V.C., Keown, M., Michaelis, J., Lawrence, J., Mateen, A., Allende, L., Eagles, P.A., et al.: Pluripotent protective effects of carnosine, a naturally occurring dipeptide. *Ann N Y Acad Sci* 854:37-53, 1998
12. Hou, W.C., Chen, H.J., and Lin, Y.H.: Antioxidant peptides with Angiotensin converting enzyme inhibitory activities and applications for Angiotensin converting enzyme purification. *J Agric Food Chem* 51:1706-1709, 2003
13. Parving, H.H., Lehnert, H., Brochner-Mortensen, J., Gomis, R., Andersen, S., and Arner, P.: [Effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes]. *Ugeskr Laeger* 163:5519-5524, 2001
14. Brenner, B.M., Cooper, M.E., de Zeeuw, D., Keane, W.F., Mitch, W.E., Parving, H.H., Remuzzi, G., Snapinn, S.M., Zhang, Z., and Shahinfar, S.: Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 345:861-869, 2001
15. Martoglio, B., and Dobberstein, B. Signal sequences: more than just greasy peptides. *Trends Cell Biol* 8:410-415, 1998

16. von Heijne, G.: Signal sequences. The limits of variation. *J Mol Biol* 184:99-105, 1985
17. Freedman B.I., Hicks P.J., Sale M.M., Pierson E.D., Langefeld C.D., Rich S.S., Janssen B., Yard B.A., Van der Woude F.J. and Bowden D.W.: A Leucine Repeat in the Carnosinase Gene CNDP1 is Associated with Diabetic Nephropathy in European Americans. *Nephrol Dial Transplant.* ;22(4):1131-1135, 2007. Epub 2007 Jan 5
18. Zschocke J., Nebel A., Wicks K., Peters V., El Mokhtari N.E., Krawczak M., van der Woude F., Janssen B. and Schreiber S.: Allelic variation in the CNDP1 gene and its lack of association with longevity and coronary heart disease. *Mech Ageing Dev.* 127:817-820, 2006
19. Teufel, M., Saudek, V., Ledig, J.P., Bernhardt, A., Boularand, S., Carreau, A., Cairns, N.J., Carter, C., Cowley, D.J., Duverger, D., et al.: Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. *J Biol Chem* 278:6521-6531, 2003
20. Hebert, D.M., Garmann S.C., Molinari M.: The glycan code of the endoplasmic reticulum: asparagine-linked carbohydrates as protein maturation and quality-control tags. *Trends Cell Biol* 15:364-370, 2005
21. Hou W.C., Chen H.J., Lin Y.H.: Antioxidant peptides with Angiotensin converting enzyme inhibitory activities and applications for Angiotensin converting enzyme purification. *J Agric Food Chem.* 51(6):1706-1709, 2003
22. Park Y.J., Volpe S.L., Decker E.A.: Quantitation of carnosine in humans plasma after dietary consumption of beef. *J Agric Food Chem.* 53(12):4736-4739, 2005
23. Fleisher L.D., Rassin D.K., Wisniewski K., Salwen H.R.: Carnosinase deficiency: a new variant with high residual activity. *Pediatr Res.* 14(4 Pt 1):269-271, 1980
24. Gjessing L.R., Lunde H.A., Morkrid L., Lenney J.F., Sjaastad O.: Inborn errors of carnosine and homocarnosine metabolism. *J Neural Transm Suppl.*; 29:91-106, 1990
25. Duane P., Peters T.J.: Serum carnosinase activities in patients with alcoholic chronic skeletal muscle myopathy. *Clin Sci (Lond).* 75(2):185-190, 1988

**Table 1:**

Signal sequence of the *CNDP1* cDNA clone

... 5' ATGGATCCCAAACTCAGGAGAARGGCTGCGTCCCTGCTGGCTGTGCTGCTGCTGCTGCTGCTGGAGCGCGGCATGTTCTCC  
3' ...

Reverse Primer for all steps: 5' AGCTTTAAATCGATGGAGCTGGGCCATCTCTAA 3'

1.PCR Fwd Primer	2.PCR Forward Primer	3.PCR Forward Primer/Tm
5' <u>(I)</u> GAGCG CGGC 3'	5' ATGGATCCCAAAC <u>T</u> CAGGAGAATGGCTGCGTCCCTG CTGGCTGTG <u>(I)</u> GAGCGCGGC 3'	5' CAGCCCATCGATATGGATCCCAAAC <u>T</u> C <u>(II)</u> GGAGA 3'
(I) Length of leucine repeat: for 4L (I) is "CTGCTGCTGCTG", for 5L add "CTG", for 6L add "CTGCTG", for 7L add "CTGCTGCTG" and for 8L add "CTGCTGCTGCTG"		
(II) A/G variant: for the arginine-polymorphism add "A" at this position, "G" for the glycine-polymorphism		

## LEGEND TO THE FIGURES:

**Figure 1:** Western blot analysis of cell protein (C) and supernatant (S) of Cos-7 cells, transfected either with 6L (lane a) or empty vector (lane b). Immunostaining with anti-CNDP1 antibody (K18K, left) and anti-myc (right) antibody is shown. Immunostaining with anti-CNDP1 of R&D showed similar results (blot not shown).

**Figure 2:** Analysis of N-glycosylation of carnosinase expressed in cell extracts and supernatants. Aliquots of cell extract and supernatant of transfected cells (6L) were either (+) or not (-) treated with PNGase to remove N-glycosyl residues. Note that after PNGase treatment the molecular weight of carnosinase in supernatants and cell extracts were equal, whereas this was not found in untreated samples.

**Figure 3:** A: Representative Western blot analysis of supernatant (S) and cell protein (C) obtained from Cos-7 cells transfected with *CNDP1* variants containing 4, 5, 6, 7 and 8 leucine repeats (4L to 8L).  
B: Analysis of secretion efficiency. The expression of carnosinase in supernatant and cell extracts was measured by densitometry. The results are expressed as mean percentage secretion  $\pm$  SD. The number of transfections used in this analysis for each of the *CNDP1* variants was: n= 7, 8, 8, 7 and 3 for 4L, 5L, 6L, 7L and 8L respectively.

**Figure 1**

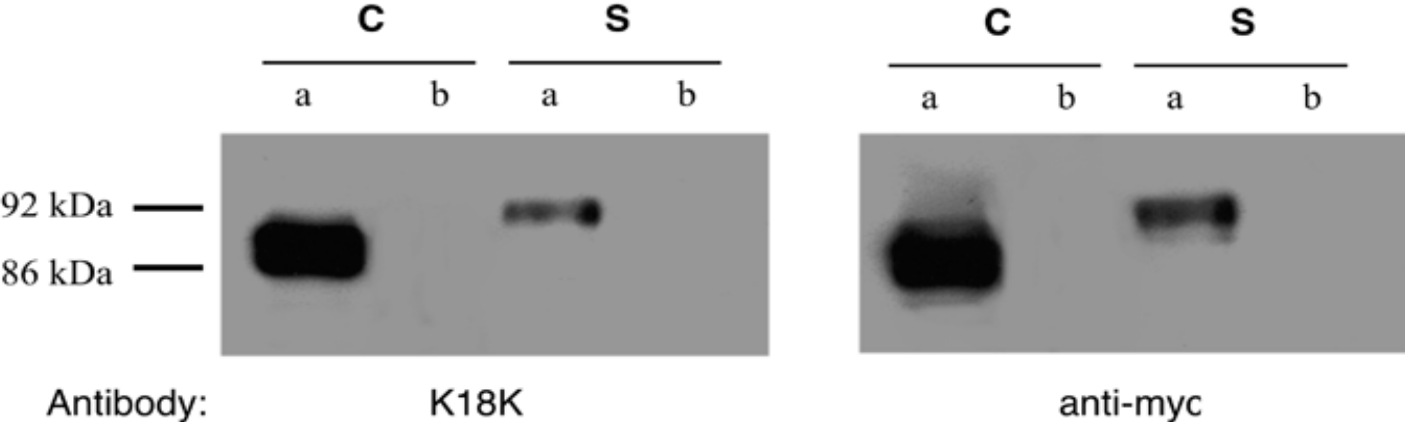
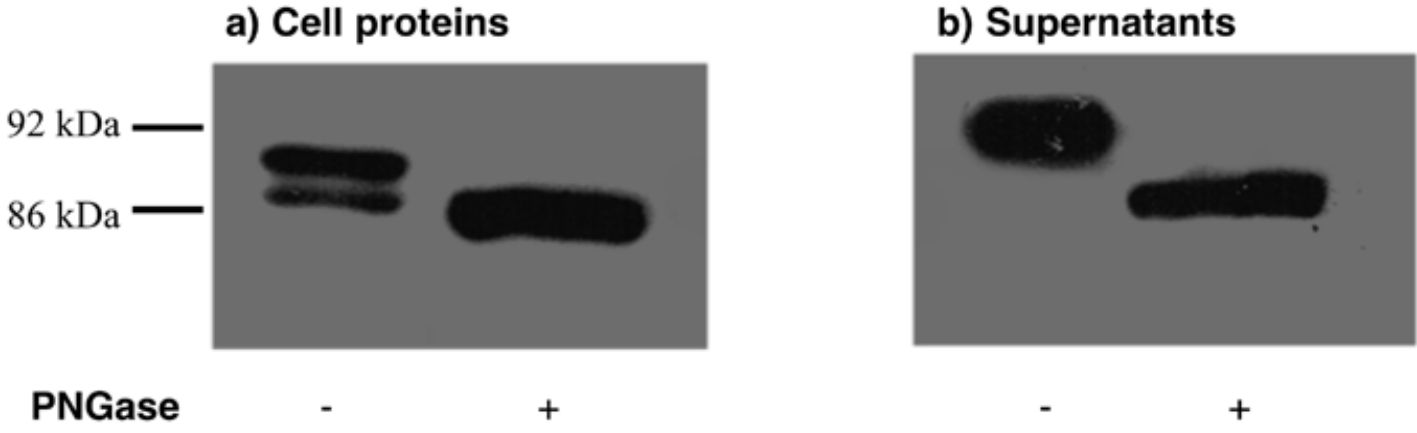


Figure 2



**Figure 3A**

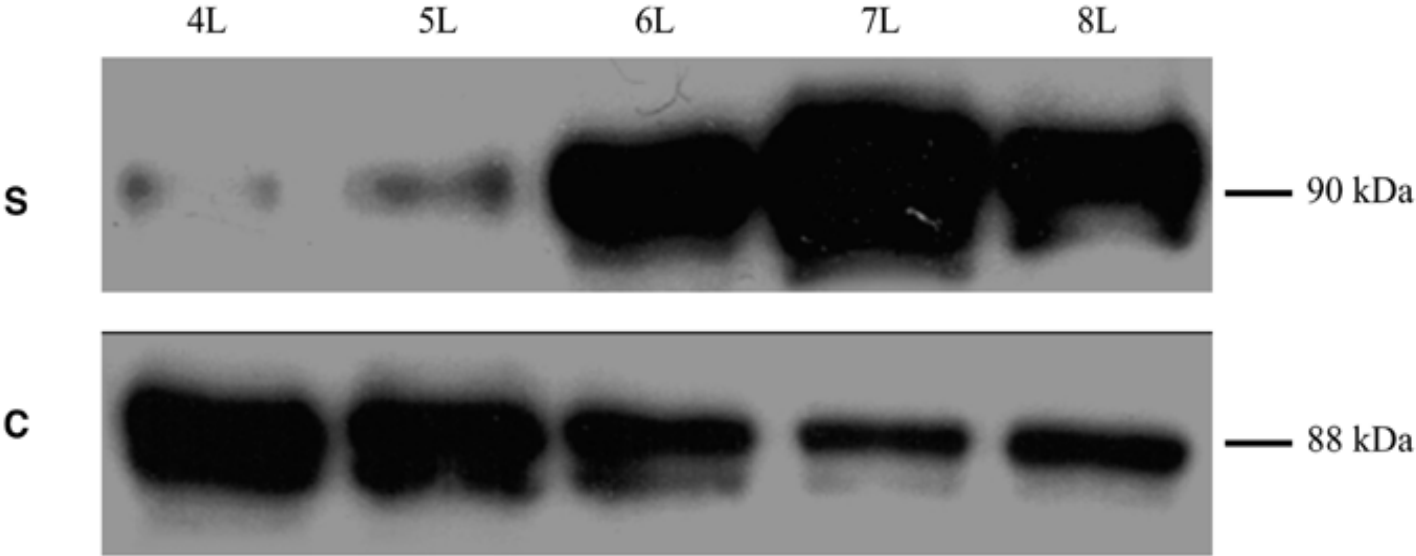


Figure 3B

