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C1q Domain Interactions in Otolith Morphogenesis

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Abstract

Otoliths ("ear stones") in fish and its homologous otoconia ("ear dust") in higher vertebrates are essential in detecting linear acceleration, as well as providing a sense of balance. They consist of mainly $CaCO_3$ which interweaves with proteins to form a matrix. The investigation of otolith morphogenesis allows proteins forming the matrix to be identified and characterized. The recently identified precerebellin-like protein (Cblnl) in trout otolith has a C1q domain that is capable of interacting with the C1q domain of Otolin-1 (Otol1a) in zebrafish otolith. It is proposed that the C1q domain of Otol1a forms homotrimers, and heterotrimers with that of Cblnl. By performing site-directed mutagenesis, we successfully deleted the two introns in trout Cblnl genomic clone to create a cDNA, which is now available for subcloning into a bacterial expression vector. We then expressed the recombinant Otol1a C1q domain via pET system and carried out affinity purification under denaturing conditions. Isolated target proteins were then refolded by dialysis in urea step gradient buffers and a fractional matrix with various reagents at different concentrations.

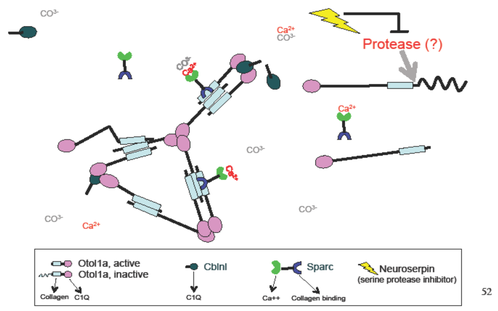
Introduction

Otoliths ("ear stones") in the inner ear of zebrafish are homologous to the otoconia of mammals, playing significant roles in linear acceleration detection and balancing. They consist of a meshwork of proteins interspersed with $CaCO_3$ crystals. The secretion and assembly of the organic matrix into the endolymph provides a template for biomineralization, thereby initiating otolith calcification in daily ring-like increments.[1]

Collagens – large domains of repeating Gly-X-Y sequences folded into a triple-helical structure – are present in Otolin-1 (Otol1a), a type of matrix protein in zebrafish otolith. These collagens in Otol1a are related to the Collagen VIII and X family and are essential for the structural organization of the matrix prior to calcification via multimer assembly. They exist in association with a C1q domain, which is also found in the Precerebellin-like protein (Cblnl) in trout otolith; however, instead of the collagen-like region, Cblnl has a homologous NC1(X) domain that is capable of trimeric or higher order multimer assembly without adjacent triple helical segments.[2]

Hence, we propose that the C1q domain of Otol1a forms homotrimers and interact with that of Cblnl during otolith framework assembly. Trimers of C1q domain form hubs while collagen fibrils (triple helices) form spokes, assembling the short chain collagens into a reticular network of matrix.[3]

A Collagen Framework in Otolith Formation?



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Objectives

- Delete the two introns in trout Cblnl genomic clone to create a cDNA.
- Express Otol1a C1q domain and affinity-purify under denaturing conditions and decreasing pH.
- Assess solubilization and refolding of Otol1a C1q domain.

Materials and Methods

Creating a cDNA by deleting the two introns in trout Cblnl genomic clone

- Extension of mutagenic oligonucleotide primers by PCR
- DpnI digest to eliminate original template DNA
- Preparation and transformation of electrocompetent DH5α E. coli via electroporation
- Screening of colonies with mutagenized plasmids by restriction digestion and DNA sequencing

Expressing and purification of recombinant Otol1a C1q domain

- cDNA for Otol1a C1q domain with His_6 tag
- Culture of host cells with T7 RNA polymerase gene (pET system)
- Expression of C1q domain via IPTG induction
- Column affinity chromatography under denaturing conditions and steps of decreasing pH

Investigating the solubilization and refolding of recombinant Otol1a-His_6

- Dialysis in urea step gradient buffers and cross-linking with BS3
- Pierce Protein Refolding kit

Figure 1 shows the experimental process.

Results

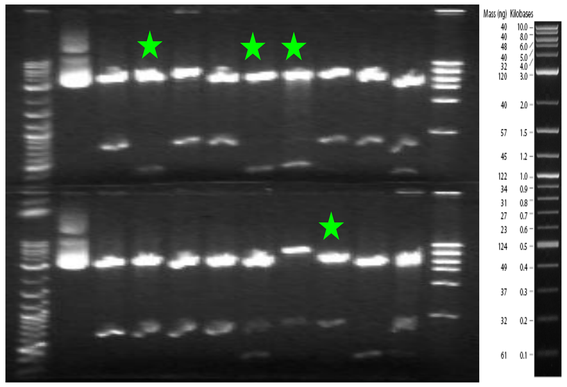


Figure 2: XhoI and EcoRI double digest products separated on 1% agarose gel. Upper section: primers set 1; lower section: primers set 2. Lanes 1&12: 2-log and Hi-Mass size markers (key at right panel); Lanes 2&3: uncut&cut mutagenesis template; Lanes 4-11: Different colonies from mutagenesis with template, primers, DNA polymerase and DpnI. Lanes with stars showed colonies with insert of expected size (~0.5 kb).

Results

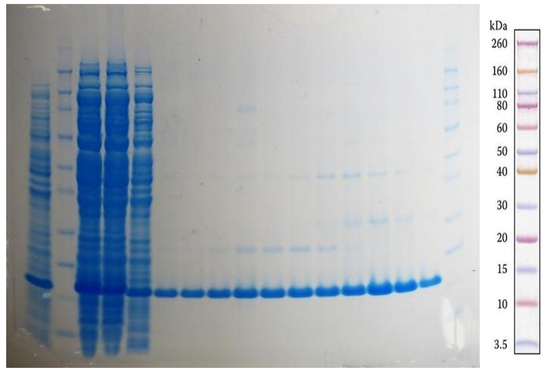


Figure 3 shows Coomassie stained SDS-PAGE image of column affinity purification of Otol1a C1q domain. Lanes 2&17: size markers (key at right panel); Lane 1: total cell protein; Lane 3: cell lysate; Lane 4: Flow-through; Lane 5: Wash at pH 8.0; Lane 6: Wash at pH 6.8; Lanes 7-16: Fractions 7-16 eluted with pH gradient (6.8-4.5) followed by pH 4.5. Fractions 7-16 showed Otol1a C1q domain of expected size (~15 kDa), present in all fractions due to overexpression. Fraction 14 showed the most dense band indicating the highest concentration of C1q domain, along with additional bands at ~30 kDa and ~50 kDa, suggesting the presence of dimers and trimers.

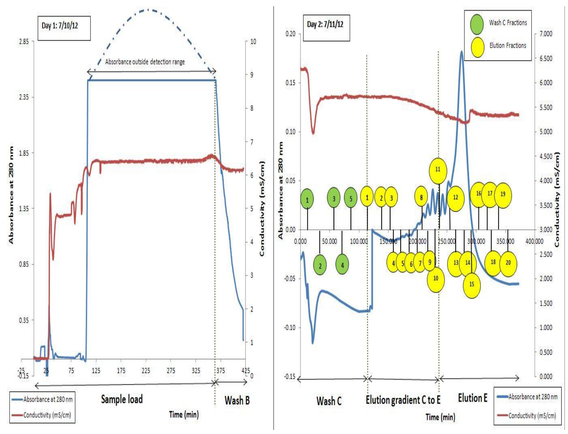
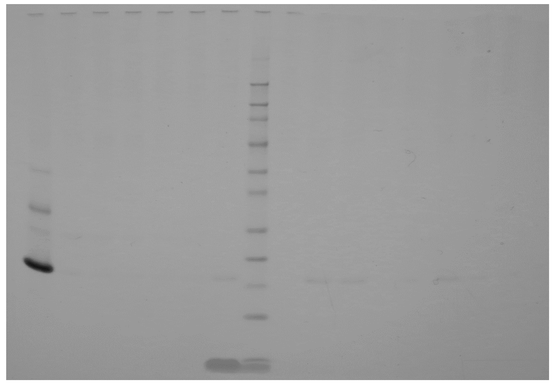


Figure 4: Chromatogram of column affinity purification of Otol1a C1q domain during day 1 and 2, with fractions indicated. Absorbance plateaued during sample load due to high amounts of overexpressed proteins. Day 2 showed sharp increase in absorbance at fraction 14 during elution at pH 4.5.



Results

Figure 5 (bottom left) shows Coomassie stained SDS-PAGE image of dialysis with urea step gradient buffers & cross-linking with BS3, and Pierce Protein Refolding kit reactions of purified Otol1a C1q domain. Lane 8: size marker; Lane 1: dialysate; Lane 2: supernatant of dialysate; Lanes 3-6: cross-linking reactions using BS3/C1q molar ratios of 1, 10, 100, 1000 (respectively); Lane 7: +ve control-purified C1q in buffer E (8M urea, pH 4.5); Lanes 9-17: Different Pierce buffers reactions. Cross-linking reactions showed faint stains at ~17 kDa due to diluted C1q concentration as a result of dialysis. More sensitive techniques such as Silver stain and Western blot should be employed for further analysis. Pierce reactions showed stains at ~17 kDa with Pierce buffers 2 and 3 (Lanes 10&11) containing L-arginine. Pierce buffers 4-9 (Lanes 12-17) contained guanidine which precipitated out of solution during gel loading, hence for future analysis guanidine has to be removed by dialysis with phosphate buffer prior to gel loading. Pierce buffer 1 contains salts.

Conclusions

- The two introns in trout genomic DNA were successfully deleted.
- Otol1a C1q domain was expressed and purified with high yield (~40mg) and purity (~95%).
- The denatured Otol1a C1q domain was solubilized and possibly refolded in the presence of arginine.

Future Directions

- Subcloning of open reading frames into bacterial expression vector for Cblnl C1q domain, followed by expression and purification.
- Test additional adjuvants for refolding and access multimerization of Cblnl and Otol1a C1q domain.

Literature Cited

1. Murayama, E., Takagi, Y. & Nagasawa, H. (2003). Immunohistochemical localization of two otolith matrix proteins in the otolith and inner ear of the rainbow trout, Oncorhnchus mykiss: comparative aspects between the adult inner ear and embryonic otocysts. *Histochem Cell Biol* 121:155-166.
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