Development of a Novel Carrier Protein for Monoclonal and Polyclonal Antibody Production

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ABSTRACT

Background: Carrier proteins are critical in the antibody production process as they confer immunogenicity to poorly immunogenic compounds such as small molecules or peptides. Hemocyanins are copper containing proteins used for oxygen transport in arthropods and mollusks and are routinely utilized as carrier molecules due to their size, potent immunogenicity, and phylogenic distance from mammalian hosts/antigens. The most commonly used carrier protein is keyhole limpet hemocyanin (KLH). However, current immunization protocols often require alternating carrier molecules to prevent immunodominance of the carrier to the detriment of the antibody response to the hapten. Thus, there is a need for an alternative highly immunogenic carrier protein. Lobster hemocyanin (LLH), is readily available as a byproduct from the food industry. Here, we evaluate the utility of lobster hemocyanin as a carrier protein of hapten antigens in comparison with KLH. Methods: Hemocyanin was purified from American lobster (Homarus americanus) serum. Peptide, protein and hormone antigens were covalently conjugated to LLH and KLH. New Zealand white rabbits and/or BALB/c mice were immunized with either the KLH-antigen conjugate or the LLH-antigen conjugate. Serum was collected at designated time points following immunizations and antigen specific antibody titers were measured by indirect ELISA. Antibody responses directed against the carrier molecule (LLH or KLH), as well as cross-reactivity of anti-LLH and anti-KLH antibodies, were also measured via indirect ELISA. Results: Pre-existing antibodies against LLH were undetectable by ELISA in sera isolated from naïve rabbits. Immunization with both LLH and KLH conjugated antigens resulted in the production of antigen specific antibodies. There was no significant difference in the antibody titers generated against the peptide or protein antigens conjugated to LLH or KLH. Conclusions: LLH is an immunogenic and effective carrier protein that promotes the generation of polyclonal antibodies to conjugated haptens as effectively as KLH. Anti-LLH antibodies do not cross-react with KLH, signifying that the LLH epitopes are unique from those on KLH. Thus, LLH is novel carrier protein that can be used as an alternative to or in conjunction with KLH.

INTRODUCTION

Figure 1. Structure of lobster hemocyanin. Lobster Hemocyanin has 3 isoforms, which form hexamers (1 x 6mers) or multiples of hexamers (e.g. 2 x 6).

Small antigens such as low molecular weight proteins, peptides, and drugs, are not innately immunogenic and must be conjugated to larger, more complex molecules for successful generation of antibodies against these haptens. Hemocyanins, proteins used for oxygen transport in arthropods and mollusks, are routinely utilized as carrier molecules due to their size, potent immunogenicity, and phylogenic distance from mammalian hosts/antigens1. The most commonly used carrier protein is isolated from the mollusk keyhole limpet hemocyanin (KLH), and forms multi-meric structures (45Mda-130Mda). Due to its size, KLH also can aggregate and precipitate, making it difficult to conjugate. By contrast, hemocyanin derived from lobsters and other arthropods are composed of smaller subunits (76-78kDa) that form a hexameric structure (Figure 1)2,3. Current immunization protocols often require alternating carrier molecules to prevent immunodominance of the carrier to the detriment of the antibody response to the hapten. Thus, there is a need for an alternative highly immunogenic carrier protein. Lampire's lobster hemocyanin (LLH), is readily available as a byproduct from the food industry for which over 66 metric tons of lobsters were harvested in 20154 (NOAA, 2015). LLH maintains several of the same benefits as KLH including phylogenetic distance from mammalian hosts and multiple available lysines (33) for coupling haptens5 (Kusche, 2001). Here, we evaluate the ability of Lobster hemocyanin as a carrier protein of hapten antigens in comparison with KLH.

RESULTS

Purification of Hemocyanin from Lobster serum

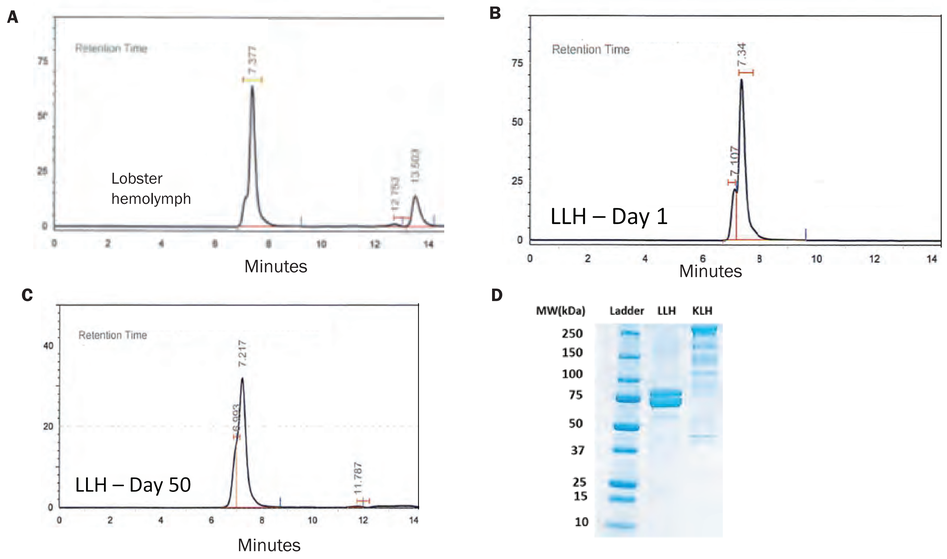


Figure 2. Purification of Lobster Hemocyanin

Hemolymph was purified from American lobster hemolymph (Homarus americanus, provided by Lobster Unlimited, Inc.). Raw lobster hemolymph and purified LLH were analyzed by HPLC (A-C) and reduced SDS-PAGE (B). (A) Lobster hemolymph contains multiple protein bands with retention times of 7.3 and 13.5 min. (B-C) Following purification, LLH was detected as one major peak with a retention time of 7.3 ± 0.1 minutes. LLH remained stable over time as it exhibited the same signal after 50 days at 2-80C (C). (D) LLH (4 ug) resolved into 2 bands at a molecular weight around 75kDa. KLH resolved into a major band with a molecular weight of >250kDa.

LLH elicits a robust antibody response that does not cross-react with KLH

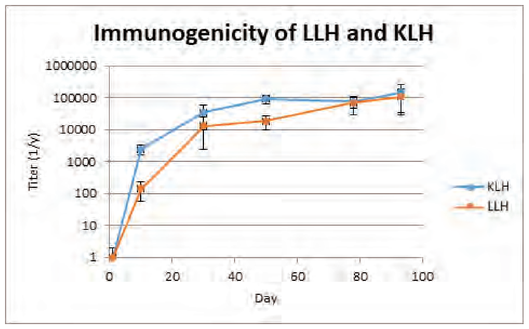


Figure 3. Comparison of Antibody titers between LLH & KLH.

The immunogenicity of the carrier molecule was determined by screening sera from Peptide-KLH or Peptide-LLH immunized rabbits against KLH or LLH respectively by ELISA (N = 3). Pre-existing antibodies against LLH were undetectable by ELISA in sera isolated from naïve rabbits. Antibody titers generated against LLH were consistently less than that generated against KLH, however these differences were only significant at the day 10 and day 50 time points (2 Tailed Students T-test; **0.05 > p ≥ 0.01; ***p < 0.01).

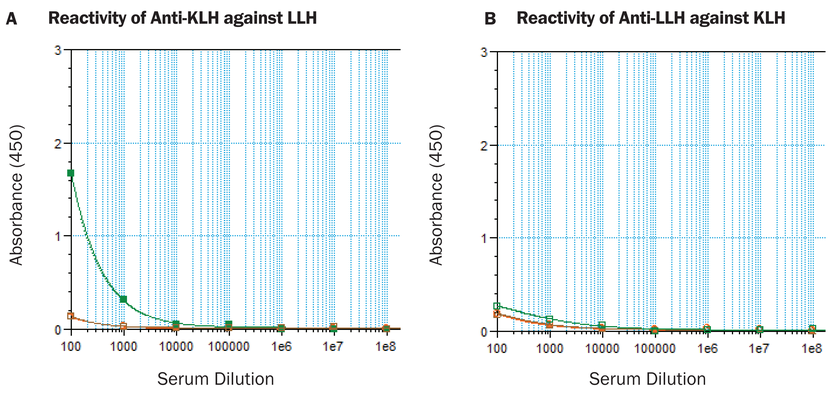


Figure 4. Cross-reactivity of LLH and KLH specific antibodies

For use in rotating, co-immunization protocols, it is necessary that antibodies elicited by LLH do not cross react with KLH and vice versa. Serum from rabbits immunized with KLH were screen via direct ELISA against LLH (A). LLH-specific antiserum screened against KLH via direct ELISA (B). Anti-LLH antibodies did not cross react with KLH, however sera from one animal immunized with KLH had low cross-reactivity to LLH (titer <100) (A). Shown are representative titration curves from Day 50 time point.

RESULTS

Immunization with both LLH and KLH conjugated antigens resulted in the production of antigen specific antibodies

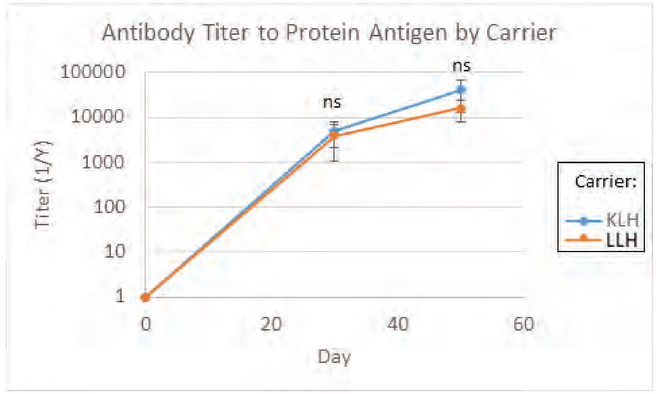


Figure 5. Immune response comparison between LLH and KLH conjugated to a protein antigen.

Rabbits (n=3) were immunized with a synthetic peptide that had been coupled with KLH or LLH. Serum samples were collected immediately before immunization, 30 and 50 days post-immunization and then screened in a protein-specific, direct ELISA procedure. Titers were not significantly different between the two carriers.

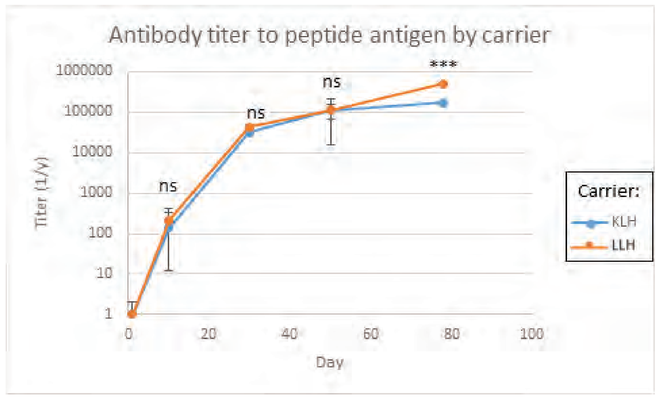
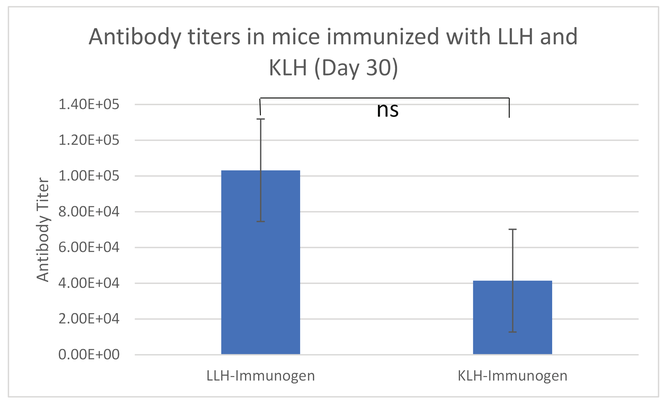


Figure 6. Immune response comparison between LLH and KLH conjugated to a peptide antigen.

Rabbits (n=3) were immunized with cross-linked peptide (no carrier) or peptide that had been coupled with KLH or LLH. Serum samples were collected immediately before immunization, 10, 30, 50 and 78 days post-immunization and then screened in a peptide-specific, direct ELISA procedure. Titers were not significantly different between the two carriers, except for the day 78 timepoint (2 Tailed Students T-test; ***p < 0.01).

Monoclonal antibody generation with LLH.



	LLH-Immunogen	KLH-Immunogen
Fusion positivity	+	+
>3 clones isolated?	YES	YES

Figure 7. Monoclonal antibody generation with LLH.

Mice (N=3) were immunized with a hormone conjugated to LLH or KLH and antibody response to the hormone was determined by ELISA (A). Spleens from the highest responding mice were removed and splenocytes were fused to SP2 cells. Fused cells were screened for reactivity to the hormone via ELISA (1-15 positives +, 16-30 positives ++, >31 positives +++). These parents were sub-cloned via limiting dilution. Greater than 3 clones were isolated for each immunogen.

CONCLUSION

- LLH is an effective carrier protein for monoclonal and polyclonal antibody generation as there is no pre-existing immunity to LLH in rabbits or mice, and comparable antibody titers are elicited between LLH and KLH conjugated protein and peptide antigens.
- Anti-LH antibodies do not cross-react with KLH, thus LLH could be alternated in immunization protocols to reduce the immunodominance of the carrier molecule.
- LLH is less immunogenic than KLH, which may be a benefit in situations where the antibody response to the carrier molecule overwhelms the response to the hapten; e.g long immunization protocols, poorly immunogenic antigens such as steroids or drugs.

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