Isolation and expression of allergens from midges (*Culicoides* spp) causing insect bite hypersensitivity in horses

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Thesis for the degree of Master of Science
University of Iceland
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HÁSKÓLI ÍSLANDS

Einangrun og tjáning ofnæmisvaka úr smámýi (*Culicoides spp*) sem orsakar sumarexem í hestum

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Ritgerð til meistaragráðu í lífeindafræði

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Ágrip

Sumarexem er ofnæmi af gerð I gegn próteinum smámýs sem lifir ekki hér á landi. Ofnæmið er algengt í útfluttum hestum, um helmingur þeirra sem hafa verið tvö ár eða lengur á flugusvæðum fá sumarexem. Íslenskir hestar fæddir erlendis fá ofnæmið í minna mæli. Einangruð hafa verið 15 ofnæmisvakagen úr flugnabitkirtlum og próteinin tjáð í *E. coli*. Tveir þessara vaka eru Cul nub a21 og Cul nub c4. Cul nub a21 skráir fyrir óþekktu próteini en inniheldur hneppi (domain) sem hefur samsvörun við svokölluð penaeidin-peptíð sem eru flokkur cysteinríkra, sýkladrepandi peptíða í rækjum. Cul nub c4 er röð sem var veidd upp úr *Culicoides nubeculosus* yfirborðsfögugenasafni með bindingu próteinsins við IgE úr sumarexemhestum. Röðin hefur samsvörun við röðina EU978914 sem skráir fyrir óþekktu próteini CNSG79. Nauðsynlegt er að framleiða ofnæmisvakana í skordýrafrumum til þess að þeir séu rétt sykraðir og sem líkastir þeim náttúrulegu. Markmiðið var að einangra og raðgreina að fullu Cul nub a21 og Cul nub c4, tjá þau í skordýrafrumum og hreinsa a21 próteinið. Einnig að setja V5 tjáningarmerki á pFastBac HT B ferju sem er notuð við tjáningu próteina í skordýrafrumum.

V5 merkihali úr pcDNA3.1/V5-His B ferju var settur inn á pFastBac HT B og virkar vel í próteingreiningu. Genin voru mögnuð upp úr cDNA lambdasafni gerðu úr bitkirtlum *C. nubeculosus*, límd inn í pFastBac HT B-V5 og pFastBac HT B og síðan raðgreind, a21 er 1218 basapör (405 amínósýrur) en c4 1254 basapör (418 amínósýrur).

Cul nub a21 og Cul nub c4 voru tjáð í Sf-9 skordýrafrumum með Baculoveirukerfi. Endurraðaðar baculoveirur voru efldar og títreraðar og próteinraðbrigðin magnframleidd og prófuð. Einnig voru framleidd fjölstofna mótefni í músum gegn *E. coli* framleiddum a21 og c4 hlutapróteinum. Bæði próteinin tjáðust í innlyksum í Sf-9 frumunum. Í ónæmisþrykki var a21 raðbrigðið 53 kDa greint með fjölstofna músamótefnunum en a21-V5 55 kDa greint með V5 sérvirkum mótefnum. Músamótefnin sem framleidd voru gegn c4 hlutapróteininu bundu ekki skordýrafrumuframleidda c4 raðbrigðið, en c4-V5 sýndi 57 kDa band í ónæmisþrykki. Mögulegt reyndist að hreinsa eðlissvipt a21 með His-Select Nickel affinity geli.

Áríðandi er að framleiða ofnæmisvakana sem líkasta náttúrulegu próteinunum þar sem sykrun getur ráðið miklu um ofnæmisvirkni. Einnig er hentugt að hafa hreinsaða ofnæmisvaka úr mismunandi framleiðslukerfum til að vinna með í ónæmismeðferð á sumarexemi. En a21 *E. coli* raðbrigðið er einn af þeim ofnæmisvökum sem byrjað er að nota í bólusetningartilraunum á hestum.

Abstract

Insect bite hypersensitivity (IBH) of horses is an IgE mediated allergic reaction to proteins from salivary glands of biting midges (*Culicoides spp*.). These insects are not indigenous to Iceland. All horse breeds can get IBH, but it is especially common in Icelandic horses exported from Iceland. If not protected against the midges they get IBH at a frequency of 50% after two years or more in *Culicoides* infested areas. Icelandic horses born abroad get IBH at a much lower frequency. Fifteen allergens have been isolated from salivary glands and expressed in *E. coli*. Two of these allergens are Cul nub a21 and Cul nub c4. Cul nub a21 codes for an unknown protein containing a domain homologous to penaeidin peptides, a group of cysteine rich, antimicrobial peptides in shrimps. The Cul nub c4 gene sequence was deduced from a phage surface display cDNA library based on the binding of the c4 protein to IgE from horses with IBH. The nucleotide sequence is homologous to EU978914 coding for the protein CNSG79. It is important to produce the allergens in insect cells to get correct glycosylation and folding. The aim of this study was to isolate and sequence Cul nub a21 and Cul nub c4, express the proteins in Sf-9 insect cells and purify Cul nub a21. Furthermore to ligate a V5 tag into the pFastBac HT B vector, used for protein expression in insect cells.

V5 tag from pcDNA3.1/V5-His B vector was ligated into pFastBac HT B. The genes were amplified from cDNA lambda library made from *C. nubeculosus* salivary glands, ligated into pFastBac HT B-V5 and pFasBac HT B and sequenced, a21 is 1218 basepairs (bp) (405 amino acids (aa)) and c4 is 1254 bp (418 aa).

Cul nub a21 and Cul nub c4 were expressed in Sf-9 insect cells in a Baculovirus system. Recombinant baculoviruses were amplified and the recombinant proteins produced and tested. Polyclonal antibodies were made in mice against the *E. coli* produced partial proteins a21 and c4. Both proteins were expressed in inclusion bodies in the Sf-9 cells. In Western blot (WB) the recombinant a21 was detected as a 53 kDa protein with the polyclonal mouse antibodies but the recombinant a21-V5 was 55 kDa with monoclonal V5 antibodies. The polyclonal mouse antibodies made against the c4 partial protein did not bind to the Sf-9 produced whole recombinant c4 protein, but the recombinant c4-V5 showed a 57 kDa band in WB with the monoclonal V5 antibodies. It was possible to purify denatured a21 with His-Select Nickel affinity gel.

It is of importance to produce the allergens in the natural form or at least with the correct glycosylation for testing the allergenicity. It is also useful to have purified allergens produced with different expression systems to use for immunotherapy. The recombinant a21 made in *E. coli* is one of the allergens that are now being tested in a vaccine experiment in Icelandic horses.

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Abbreviations

aa	Amino acid
APC	Antigen presenting cell
BLAST	Basic local alignment and search tool
bp	Base pair
BSA	Bovine serum albumin
Cul nub	*Culicoides nubeculosus*
E. coli	*Escherichia coli*
FBS	Fetal bovine serum
IBH	Insect bite hypersensitivity
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LB	Luria broth
NK cells	Natural killer cells
PBMC	Peripheral blood mononuclear cells
PBS	Phosphatase buffered saline
PCR	Polymerase chain reaction
Sf-9	*Spodoptera frugiperda*
SGE	Salivary gland extract
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
Sim	*Simulium vittatum*
TBE	Tris borate-EDTA
TBS	Tris buffer saline
TBS-T	Tris buffer saline with Tween 20
TGF	Tumor growth factor
Th	T helper cell
Treg	T regulatory cell
WB	Western blot

1 Introduction

1.1 Insect bite hypersensitivity in horses

Insect bite hypersensitivity (IBH) is a type I hypersensitivity, a recurrent seasonal dermatitis of horses also known as summer eczema or sweet itch. It is caused by an IgE mediated reaction against proteins from the salivary glands of *Culicoides* spp. (midges) (Baker et al., 1978; Brostrom et al., 1987; Heimann et al., 2010). In severe cases chronic symptoms are severe itching (pruritus) causing lesions, thickening of the skin, and open wounds because of self excoriation that often is followed by secondary bacterial or fungal infections (figure 1). The disease is usually localized at the mane, tail and withers area and sometimes along the belly (linea alba). The symptoms decrease or disappear in the winter but reappear in the summer. If left untreated, the symptoms can worsen during the summer period, rendering many horses unfit for riding (Baker, et al., 1978; Bjornsdottir et al., 2006; Langner et al., 2008).

It differs between regions which *Culicoides* species can be found. However, skin tests have shown that most horses react to antigens that are found in all *Culicoides* species also from those that don't live in the area (Anderson et al., 1993).

Figure 1: Clinical signs of IBH (photo: Eliane Marti, Berne, Switzerland)

1.1.1 Epidemiology

Horses of all breeds can be afflicted with IBH but the prevalence varies between countries and regions. The prevalence of IBH in Icelandic horses born in Iceland and exported to mainland Europe is more than 50% if the horses have been living in *Culicoides* infested areas for two years or more unprotected (Bjornsdottir, et al., 2006). Midges are not present in Iceland and therefore IBH has not been described in native Icelandic horses. *Simulium* spp (black flies) are the only flies that bite mammals in Iceland (Johannsson, 1988), and though black flies do not cause IBH, the majority of horses that have developed IBH respond also to *Simulium* allergens (figure 2) (Baselgia et al., 2006; Bjornsdottir, et al., 2006).

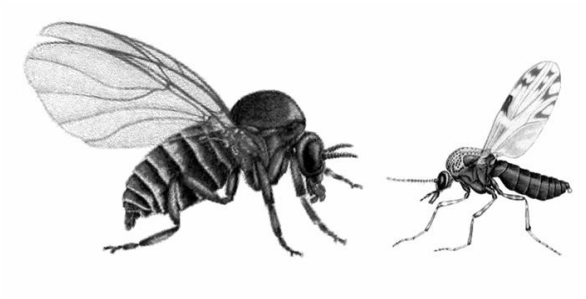


Figure 2: *Simulium spp.* and *Culicoides spp.*

There is a strong relationship between IBH and exposure to midges, dependent upon the timeline from export, habitats of *Culicoides,* pasture´s cultivation and moisture, lee at pasture and open water in the surrounding area (Bjornsdottir, et al., 2006; Brostrom, et al., 1987). The export of Icelandic horses to the mainland is followed by great environmental changes, including varying weather conditions and the introduction of new insects and microbes. The stress of the exportation process seems to have a negative influence on the immune system. Age, gender or country has no influence on the prevalence of IBH (Bjornsdottir, et al., 2006).

1.1.2 Diagnosis

Diagnosis of IBH is mostly based on physical examination and history in association with *in vivo* and *in vitro* tests. There are mainly three different test systems that can help diagnose IBH, (i) intradermal testing determines hypersensitivity by demonstrating local mast cell activation, (ii) the histamine release test utilizes horse basophilic granulocytes and (iii) ELISA measures equine serum antibodies specific for allergens and therefore determines the humoral response (Langner, et al., 2008). An *in vitro* sulphidoleukotriene release test can also be used for diagnosis of IBH. The test measures released sulfoleukotrienes after incubation of peripheral blood leukocytes with presumptive allergen extracts (Marti et al., 2008; Marti et al., 1999).

1.1.3 Treatment

Prevention against IBH is to protect the horses from biting flies (Baker, et al., 1978). Housing horses from dusk till dawn only prevents horses from exposure to *Culicoides* spp. but not *Simulium* spp. or mosquitoes, because black flies often feed during the day and the mosquitoes can enter the stables to feed (Fadok, 1990). Other solutions include keeping the horses on dry and windy pastures and the use of specific eczema blankets (figure 3). Corticoid therapy can be used to suppress symptoms (Anderson et al., 1996; Bjornsdottir, et al., 2006).

Figure 3: Horse wearing protection blanket, photo: Eliane Marti, Switzerland

1.2 The immune response

The immune response of mammals can be divided into innate and adaptive immunity, but these two systems are closely intertwined. The innate immune system provides the primary immediate defense and does not lead to immunological memory. When the innate immune response fails to eliminate a new infection it recruits the cells of the adaptive immune response. The adaptive immune system has the ability to recognize all pathogens specifically and provides enhanced protection against re-infection, based on clonal selection of lymphocytes bearing antigen-specific receptors(Janeway et al., 2005b).

Hematopoetic stem cells in the bone marrow divide to produce two types of stem cells, myeloid progenitor and lymphoid progenitor (Janeway, et al., 2005b).

The myeloid progenitor is the predecessor of white blood cells in the innate immune system; macrophages, granulocytes, dendritic cells and mast cells. Macrophages are dispersed widely in the body tissue. They are one of three types of phagocytes in the immune system, and play a critical role in the innate immune response. Monocytes are the immature form of macrophages, they circulate in the blood and differentiate into macrophages when entering the tissues. Dendritic cells are specialized in taking up antigens, processing them and displaying them to T-lymphocytes. Immature dendritic cells migrate from blood to tissues and are both phagocytic and macropinocytic, i.e. they can ingest large amount of surrounding extracellular fluid. Mast cells are not well defined, but they are generally near small blood vessels and release substances that affect vascular permeability when activated. Though they are best known for their role in allergic responses, mast cells are thought to play a role in defending mucosal surfaces against pathogens. Granulocytes divide into three cell groups; eosinophils, basophils and neutrophils. They all have relatively short lifetime and are produced in large amount in infection or inflammation. Neutrophils are the third phagocytic cell and the most important cellular component of the innate immunity. Eosinophils are important in defense against parasitic infections and basophils function is probably similar to that of mast cells (Janeway, et al., 2005b). Mast cells, basophils and activated eosinophils have high affinity FcƐ receptors which bind IgE to their

surface, enabling these cells to respond to the binding of specific antigens and releasing inflammatory mediators (Janeway et al., 2005a).

Though dentritic cells are best known as antigen presenting cells (APC) initiating the adaptive response, they do not produce IL-4 important cytokine for Th2 differentiation. Recent studies indicate that basophils and mast cells that produce IL-4 play a critical role in Th2 differentiation (Abraham et al., 2010; Galli et al., 2010; Wynn, 2009). Basophils fulfill the criteria needed to function as an APC. When exposed to antigens they express MHC class II and co-stimulatory molecules, take up antigen, process it and localize to draining lymph nodes and show it to T-cells (Mikhak et al., 2009).

The lymphoid progenitor is the predecessor of the lymphocytes and natural killer cells (NK cells) in the adaptive immune system. Lymphocytes divide into B- and T-lymphocytes. Both B- and T-cells are formed in the bone marrow but only B-cells migrate there, T-cells migrate to the thymus. B-lymphocytes mature into plasma cells that secrete antibodies. T-lymphocytes divide into CD8 cytotoxic T-cells, that kill virus infected cells, and CD4 T-helper cells, activating other cells like B-cells, NK cells and macrophages. T-helper cells then divide into Th1 cells and Th2 cells depending on what cytokines they produce. Simplified, CD4 T-helper cells respond to intracellular infections by activating macrophages with cytokine CD8 cytotoxic T-cell release, but in extracellular infections they activate B-cells to produce antibodies. NK cells are a part of the adaptive immune response and recognize and kill abnormal cells like virus infected cells and cancer cells (Janeway, et al., 2005a).

Naïve T-helper cells do not normally produce cytokines but when activated they become Th0 cells and start producing cytokines and divide into Th1, Th2, Th17 or T regulatory cells (Tregs). The pathway that the APC directs the immune response is dependent on the antigen type (figure 4). The Th1 immune response is the main defense against intracellular infections (e.g. viral) by activating macrophages and directing the production of certain antibodies. The Th2 immune response stimulates B-cells in producing antibodies and is the main defense against parasites (Janeway, et al., 2005b). The Th17 immune response is critical for mucosal and epithelial host defense against extracellular bacteria and fungi. Recently it has become apparent that the Th17 response is associated with chronic inflammation and autoimmune diseases (van de Veerdonk et al., 2009). Tregs suppress immune responses including the production of anti inflammatory cytokines, direct cell to cell contact, and modulate the activation state and function of APC. In addition to restraining autoimmunity, Tregs suppressor function features in regulating other forms of immune-mediated, and likely, nonimmune, inflammation, and affects immune responses to infection and tumor growth (Sakaguchi et al., 2009). Tregs are also able to inhibit the development of allergic Th2 responses and changes in the fine balance between allergen-specific Treg and Th2, Th1 and Th17 cells are crucial in the development and treatment of allergic diseases (Akdis et al., 2009).

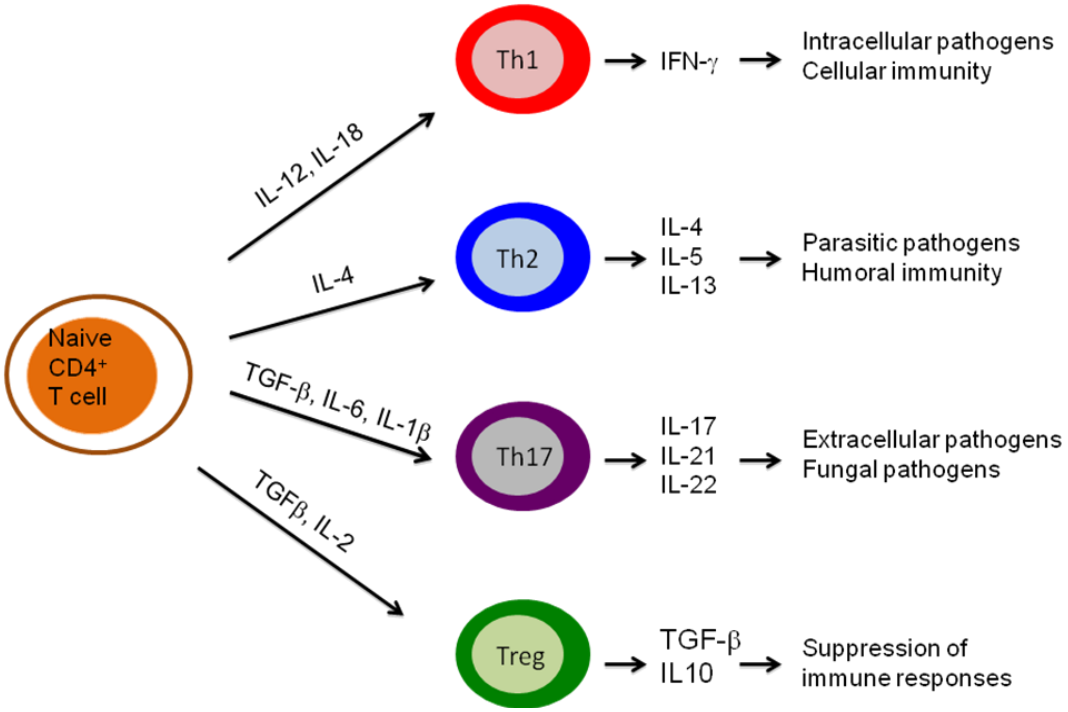


Figure 4: CD4+ T cell differentiation. Schematic picture: Sigurbjörg Þorsteinsdóttir.

Communication between lymphoid cells and non lymphoid cells takes place in specialized tissues, the lymphoid organs, and is very important for the migration of lymphoid cells and their maintenance and survival. The organs can be divided to; primary lymphoid organs; thymus, where T-cells migrate, and bone marrow where B-cells migrate, secondary lymphoid organs are the lymphoid tissue and the spleen where lymphoid cells are maintained and the activation of lymphoid cells takes place. This is where the adaptive immune response begins (Janeway, et al., 2005b).

1.2.1 Allergy

Hypersensitivity type I is an excessive or inappropriate immune reaction against otherwise innocuous antigens. At first contact the allergen provokes the production of IgE antibodies, which bind to high affinity receptors on mast cells and basophils in the exposed tissue. Cross linking of the high-affinity IgE receptors on subsequent allergen exposure leads to the activation of IgE binding cells and a series of responses that are characteristic of allergy and cause its symptoms. These include the release of short-lived mediators such as histamine and later leukotrienes, cytokines and chemokines, recruiting and activating eosinophils and basophils. Hypersensitivity type I is a Th2 mediated inflammatory disease and IL-4 or IL-13 switch B-cells to IgE production whereas IFN-γ suppresses IgE production. Hypersensitivity diseases are rarely limited to one type of hypersensitivity. Although IBH is classified as type I, other types of immunological reactions like hypersensitivity type IV or delayed hypersensitivity, mediated by T cells, cutaneous basophil hypersensitivity or IgE-mediated late phase allergic reactions may sometimes also be involved in the pathogenesis of IBH (Fadok et al., 1990; Halldorsdottir et al., 1989).

Allergic response can be divided into two phases; sensitization and reaction. Sensitization is when an allergen enters the body and is taken up by APC. Signals from the APC cause differentiation of Th2 cells and the production of IL-4, IL-5 and IL-13 drives B-cells to produce and secrete allergen-specific IgE. The IgE binds to high affinity FcƐ receptors on mast cells in tissues and on basophils in blood circulation. When the allergen enters the body again it crosslinks IgE on mast cells and basophils, they become activated and start secreting inflammatory mediators, causing allergic response (Figure 5) (Janeway, et al., 2005b; Rindsjö et al., 2010).

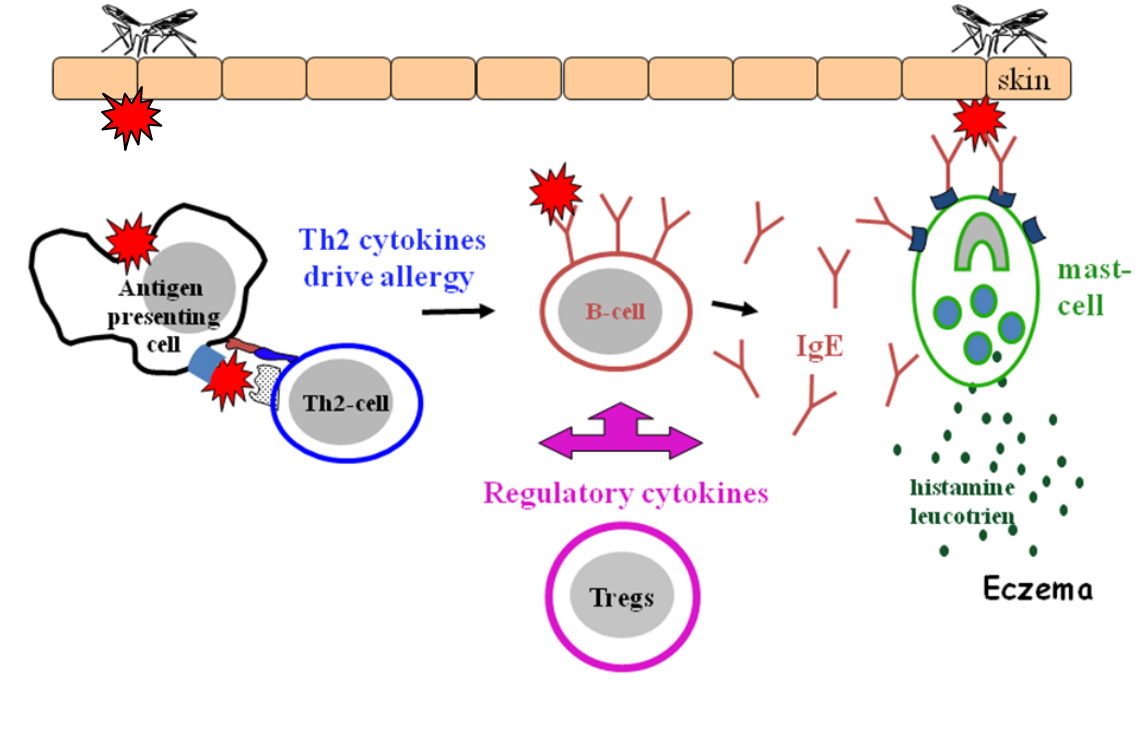


Figure 5: A simplified IgE mediated response in IBH: APC picks up an allergen from the *Culicoides* saliva and shows it to Th0 cell which differentiates into Th2 cell. The B-cell is induced by the Th2 cell to produce specific IgE antibodies against the allergen. The IgE antibodies bind FcƐ receptors on the mast cell. When a horse is bitten again the allergen crosslinks its specific IgEs on the mast cell and triggers allergic response. Schematic picture: Sigurbjörg Þorsteinsdóttir

1.2.2 The immune response of horses and the allergic response in IBH

The immune response of horses is regulated by the same cellular pathways as in other mammals. Immunoglobulins of horses are IgA, IgM, IgG, IgE and IgD. Four subgroups of IgG can be recognized with monoclonal antibodies; IgGa, IgGb, IgGc and IgG(T) (Lunn et al., 1998). Nevertheless, mapping of the gene for the heavy chain showed that IgG subgroups are up to seven. According to this, IgGa is IgG1, IgGb is IgG4, IgGc is IgG6 and IgG(t) divides into two subgroups, IgG3 and IgG5. IgG2 and IgG7 have not been found expressed as proteins (Wagner, 2006; Wagner et al., 2004). Most of the genes for the cytokines that characterize Th1, Th2 and Tregs pathways have been identified (Cunningham et al., 2003; Dohmann et al., 2000; Kato et al., 1997; Steinbach et al., 2002). In some

cases monoclonal antibodies have been raised against cytokines in horses, like anti-equine CD4, CD8, IL-4 and IL10 (Wagner et al., 2008; Wagner, et al., 2004).

It has been shown as in other animals that Th1 response with production of IFN-ɣ and IgG4 is dominating in viral infections (Breathnach et al., 2005; Svansson et al., 2009) whereas Th2 response with production of IL4, IgE, IgG1, IgG3 and IgG5 is seen in allergic diseases and in defense against parasites, especially helminths (figure 4) (Marti, et al., 1999; Mizukoshi et al., 2002; Paillot et al., 2005; Wagner et al., 2006). Viral infection is very rare in horses in Iceland but they are in general highly infected with helminths (Hamza et al., 2010; Svansson, 2004).

Because of parasitic infections, total serum IgE in healthy horses is generally very high, or up to 1000 times the normal range for humans. Horses in Iceland have higher total IgE than Icelandic horses abroad, but the IgE level decreases the first year after export and remains relatively low if the horse is not affected with IBH. In that case the IgE level increases. This has not been found in other horse breeds when total IgE is compared between IBH affected horses and healthy horses (A.D. Wilson et al., 2006).

Helminth infections are known to be associated with Th2 type immune response. Horses in Iceland had higher faecal egg counts, higher tapeworm specific IgG3/5 and higher total serum IgE levels than horses in Switzerland. However, horses in Iceland displayed a low proportion of IL-4 producing cells in peripheral blood mononuclear cell (PBMC) cultures after polyclonal or parasite extract stimulation and the addition of anti IL-10 and anti TGF-β1 to PBMC cultures of horses in Iceland increased the proportion of IL-4 producing cells after polyclonal or parasite antigen stimulation. This paralleled the high levels of IL-10 and TGF-β1 found in supernatant from PBMC cultures of horses in Iceland. Collectively, horses living in Iceland have a high parasite burden but low IL-4 production supporting the hypothesis that heavy helminth infections have a suppressive effect on IL-4 production mediated by IL-10 and TGF-β1 (Hamza, et al., 2010).

The IBH immune response *in situ* analysed by Heimann et al characterized the infiltrating cells and the cytokine milieu directly in skin lesions. The data suggest an imbalance between Th2 and Treg. IBH diagnosis was confirmed by the determination of *Culicoides*-specific IgE. In IBH lesions the total number of T-cells was increased. FoxP3 mRNA levels were reduced in lesional skin, compared to both non-lesional and to healthy skin, while the number of $FoxP3^+$ T-cells did not differ between the groups. Expression levels of IL-13 were elevated in the skin of IBH affected horses, but not IL-4 or IL-5. IL-10 levels were lower in the blood of IBH affected horses than of healthy ones. No significant changes were observed regarding blood expression levels of Th1 and Th2 cytokines or FoxP3 (Heimann, et al., 2010).

Insect bite hypersensitivity is a unique model for allergy. It presents an opportunity to compare allergenic responses between individuals with the same genetic background that on one hand are exposed to the allergy and grow up with the *Culicoides* flies and on the other hand have not encountered them.

1.3 Culicoides

Culicoides midges are the largest genus of the family Ceratopgonidae, a family of small flies in the order Diptera. They are very small, measuring about 1-5 mm at length, with a wingspan of only approx 1.4 mm and weight of approx 0.5 µg (Featherstone).

Around 1,400 species of *Culicoides* midges are known and they exist in every continent except in the polar regions, far south in South America, New Zealand and Iceland (Featherstone).

Adult midges are most active when temperatures are more than 10°C and there is little or no wind. The flies do not disperse from their breeding grounds though in a mild breeze they can be carried a kilometer or more. If wind speed goes over 8.8 km/hour the flies are unable to fly and are forced to land on the ground or trees (Blackwell, 2001; Braverman, 1994; Featherstone).

Midges are crepuscular, they are most active from dusk till dawn from May till September (Featherstone).

The midges´ lifecycle begins with an egg that is laid in summer on moist soil. The larvae hatch in a day and burrow into the soft upper layers of the soil. The larvae are both omnivorous and detrivorous, and go through 4 stages of instars (growth) separated by molts. At the final instar in the winter, the larvae have reached about 5 mm length. An increase in temperature and daylight hours activates the pupal stage for a day or two from mid May till July and then the midge emerges as a flying adult and can live for 20-30 days (Blackwell, 2001; Featherstone).

The nectar of flowers is the main energy source of the midges, but they also feed on rotting vegetation and resin. Female midges need blood after fertilization for the eggs to reach full growth, they therefore bite mammals. The choice of mammal is dependent upon species but because they are opportunists they feed on mammals they can gain access to each time (Featherstone).

When the female midge sucks blood, it cuts through the skin on its pray with specialized mandibles and mouthparts. It rolls the mouthparts into a tube and inserts them into the wound. The midge can drink for up to 4 minutes if uninterrupted. When it ceases to drink it sends out a pheromon to attract more females (Featherstone).

During blood feeding, *Culicoides* midges secrete important pharmacological compounds into the bite site to prevent coagulation and encourage vasodilation that can alter the host´s innate and acquired immune responses and become allergens in sensitive horses (Campbell et al., 2005).

1.4 Insects allergens

Stinging and biting insects are known to cause allergy in humans and animals (McDermott et al., 2000; Peng et al., 2004). The difference between biting and stinging insect is that biting insects inject venom via structures associated with the mouth, like mandibles, and stinging insect inject the venom via a tapered posterior structure called a sting. Venoms that are associated with the mouth have evolved for handling a prey and are designed to cause paralysis, widespread tissue destruction, and possibly death in the victim, while venoms that are associated with posterior stings have evolved for defense, therefore causing immediate pain. These are not fatal but the victims learn to avoid it in the

future. Both mandibles and stings are design for venom delivery and have venom glands and ducts (Vetter, 1998).

Mosquito allergy is IgE mediated and it is known that the allergens are found in the saliva. If the ducts of the salivary glands of a mosquito are cut, it can still feed and develop eggs but it cannot cause skin reactions in humans (Hudson et al., 1960; Peng, et al., 2004). There are at least eight allergens in the saliva of *Aedes aegypti* and four of them (Aed a 1-4), apyrase, α-glucosidase and two D7 proteins are major allergens (Peng, et al., 2004; Peng et al., 2006).

More than 100.000 species are in the order Hymenoptera (class: Insecta) worldwide. The main cause of allergic reactions is the venom of Aculeata (wasps, ants and bees), a division of Hymenoptera. Most anaphylactic reactions in central Europe are caused by honeybees (*Apis mellifera*) and wasps (*Vespula vulgaris, V.germanica*). Phospholipase A2, hyaluronidase, phosphatase, serine protease, allergen C and mellitin are the major allergens in bee venom. Phospholipase A2, hyaluronidase and antigen 5 are the major allergens in Vespula venom (Przybilla et al., 2010).

1.4.1 *Culicoides* and *Simulium* allergens

The allergens responsible for IBH are found in the saliva of *Culicoides.* The presence of antibodies in horse serum that recognize *Culicoides* salivary gland was demonstrated by immunohistology on section fixed *Culicoides*. Antibodies were detected in the serum of IBH affected horses and healthy horses exposed to *Culicoides* bites, but not in the serum of native Icelandic horses that had not been exposed to *Culicoides*. In both healthy and allergic horses anti-salivary IgG antibodies were detected but IgE antibodies were only detected in horses with signs of IBH (A. D. Wilson et al., 2001). At least ten IgE binding proteins in *Culicoides nubeculosus* salivary glands have been identified by Western Blotting (WB) and their size ranging from 12 to 70 kDa. Five of those proteins were suggested to be major allergens because more than 50% of horses with IBH reacted to them, and further two were recognized by 30% of the IBH horse (Hellberg et al., 2006).

Phage surface display technology has been used to identify potential allergens from *Culicoides nubeculosus* and *Simulium vittatum* associated with IBH (A. Schaffartzik et al., 2011; A Schaffartzik et al., 2009). This technique has previously been used for the isolation of allergens of humans, such as *Aspergillus fumigates, Malassezia furfur* and peanuts (Crameri et al., 2001). Phage display is a cloning system based on the linkage between the phenotype, displayed as a fusion to a phage coat protein, and the genotype, recombinant cDNA integrated into the phage genome. It allows the screening of large libraries using affinity selection for the presence of specific clones. Affinity enrichment involves phage binding to immobilized target molecules, e.g. horse IgE, during continuous rounds of selection. Hence, the selected phage displaying a protein able to connect to serum IgE, contains the genetic information as cDNA insert in the phage genome. The cDNA can be sequenced to demonstrate the amino acid sequence of the displayed gene product. This is a fast procedure and in a few days can be as much as 10^{11} single clones screened in a single well of a microtiter plate which is coated with immobilized target molecules, e.g. horse IgE (A. Schaffartzik, et al., 2011).

Fifteen potential allergens associated with IBH have been isolated from salivary glands and expressed in *E. coli* (A. Schaffartzik, et al., 2011; A Schaffartzik, et al., 2009). Four allergens from *Simulium vittatum* and 10 from *Culicoides nubeculosus* have been identified with phage surface display technology (A. Schaffartzik, et al., 2011; A Schaffartzik, et al., 2009).

According to allergen nomenclature the allergens from *Simulium* have been named r-Sim v 1, 2, 3 and 4. Sera from 25 IBH affected horses and 20 healthy control horses was analysed in ELISA and IgE levels against the r-Sim v proteins determined. It showed that IBH affected horses had significantly higher IgE levels than control horses against r-Sim v 1, 2, 3 and 4. Sixty percent of the IBH affected horses showed IgE binding to r-Sim v 1, 32% showed IgE binding to r-Sim v 2, 44% to r-Sim v 3 and 20% to r-Sim v 4. All of these allergens belong to protein families known to induce allergy in humans. rSim v 1 is homologous to antigen 5 like proteins from the mosquito genuses *Aedes* and *Culex*. r-Sim v 2 (32%) is homologous to serine protease inhibitors and r-Sim v 3 (44%) and r-Sim v 4 (20%) are homologous to α-amylases (A Schaffartzik, et al., 2009).

Ten clones with different cDNA from the salivary glands of *C. nubeculosus* were found with phage display and are candidate allergens causing IBH. The allergens were named Cul n 2-11 and sequence analyses showed strong homology to known salivary gland proteins from *C. nubeculosus* and *C.sonorensis* (A. Schaffartzik, et al., 2011). The recombinant proteins Cul n 2 to Cul n 11 showed all IgE binding above background when tested with sera of IBH affected and healthy control horses in ELISA. WB analyses with pool of five sera from IBH affected horses demonstrated specific IgE binding to the recombinant Cul n allergens. *In vitro* analyses showed that they were all able to bind serum IgE from IBH affected horses but not healthy ones. IgE sensitisation frequency against the single salivary gland allergens varies between 13 to 57% among the horses suffering from IBH. The Cul n 2 allergen has the highest frequency for IgE sensitisation, 56.7%, 47.8% showed IgE binding to Cul n 3, 45.7% to Cul n 4, 43.5% to Cul n 5 which is homologous to the Cul nub a21 described in this thesis, 34.8% to Cul n 6, 30.4% to Cul n 7, 21.7% to Cul n 8, 26.1% to Cul n 9, 15.2 % to Cul n 10 which is homologous to the Cul nub c4 also described in this thesis and 13.0% to Cul n 11 (A. Schaffartzik, et al., 2011). *In vivo* testing with eight of the recombinant proteins showed elicited immediate wheal reactions after 30 minutes in IBH affected horses and not in healthy control horses. The reaction wheals consisted after 4 hours and in some cases they increased. To confirm that these proteins are important salivary gland proteins associated with IBH the number of horses reacting with at least one of the eleven allergens (Cul n 1 included) was calculated and the recombinant allergens detected 45 of the 46 IBH affected horses, resulting in a diagnostic sensitivity of 98% (A. Schaffartzik, et al., 2011).

Some of the Cul n 2-11 allergens showed homologies to protein families which are known to induce allergen in humans; e.g. Cul n 2 codes for hyaluronidase, an allergen in bee venom (Soldatova et al., 1998), Cul n 3 has homology to cystein proteases, a major allergen of house dust mites (Shakib et al., 2008), and Cul n 8 codes for maltase, known to cause allergic reactions in humans and is also thought to be a major allergen in IBH (Langner et al., 2009). Cul n 9 shares a sequence similarity to a member of the D7-related proteins present in the saliva or salivary glands of numerous female blood-sucking insects, i.e. D7 protein in *Aedes aegypti* is involved in mosquito allergies in humans (Malafronte et al., 2003).

One allergen from *Culicoides nubeculosus,* antigen 5 like allergen named cul n 1, was isolated from λcDNA salivary gland library of *Culicoides nubeculosus* (Björnsdóttir, 2008).

Schaffartzik et al (2010) demonstrated that a homologous allergen, antigen 5 like, present in the salivary glands of *S. vittatum* and *C. nubeculosus*, shows extended cross-reactivity *in vitro* and *in vivo*. The cross-reactivity of the recombinant proteins r-Sim v1 and rCul n 1 were tested by WB analyses, inhibition ELISA and intra-dermal skin test. In WB both proteins bound strongly to serum IgE from IBH affected horses, demonstrating the recombinant proteins allergenic nature. Specific IgE against the antigen 5 like proteins of both insects was detected in 35% of the IBH affected horses. Inhibition ELISA showed that Cul n 1 in the fluid phase strongly inhibits the binding of serum IgE to solid phase coated Sim v 1 in a concentration dependant manner and vice versa. This shows that the allergens share common IgE binding epitopes. Intradermal skin test with these allergens showed immediate and late phase reactions to the IBH affected horses whereas healthy control horses do not develop relevant immediate hypersensitivity reactions (A. Schaffartzik et al., 2010).

1.4.2 Cul nub a21 and Cul nub c4 allergens

The isolation and production of Cul nub a21 and Cul nub c4 is described in this thesis. Both of them had been found by phage surface display technology by our co-workers in Switzerland.

The Cul nub c4 protein is homologous to the CNSG79 protein (acc.number: ACM40903) thought to be an enzyme with unknown function (Russell et al., 2009).

The Cul nub a21 nucleotide sequence is homologous to the published sequence from *C.sonorensis* AY603639 coding for an unknown protein. It contains a domain homologue to penaeidin peptides (Campbell, et al., 2005; Sigurðardóttir, 2008).

1.4.2.1 Cul nub A21 allergen and its relativity to penaeidin peptides

Penaeidin peptides are a group of cysteine rich, antimicrobial peptides (figure 6). Antimicrobial peptides are involved in the innate immune system of vertebrate, invertebrate and plant species for protection against microbes. Penaeidin peptides were first identified from two crustacean species, the shrimp *penaeus vannamei* and the crab *carcinus maenas* (Cuthbertson et al., 2007; Destoumieux et al., 1999).

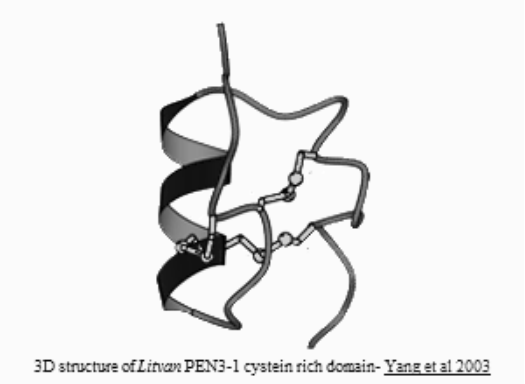


Figure 6: Penaeidin peptide. Photo: from the website http://penbase.immunaqua.com/structural_prop.php?page=prop&part=structure

2 Aims and background of the study

Insect bite hypersensitivity is an allergy in horses against salivary gland proteins from midges, *Culicoides spp.* It is very common in Icelandic horses exported from Iceland; about 50% get IBH after two years or longer in *Culicoides* infested areas if not protected against the flies. This project is a part of a bigger project, “Insect bite hypersensitivity in horses”, ongoing at Keldur from the year 2000, in collaboration with the Veterinary faculty of the University of Berne. The aim of the IBH project is to find and analyze the proteins that cause the allergy, investigate the immune response and develop a curative or preventive immune therapy for IBH. Fifteen allergens have been isolated from salivary glands, four from *Simulium vittatum* and eleven from *Culicoides nubeculosus*. All fifteen have been expressed in *E. coli* and they bind IgE from IBH affected horses. To obtain the correct glycosylation and folding, it is of importance to express the proteins in insect cells to register the true allergenicity and subsequently for employing them when developing the immune therapy

The aim of this study was to isolate and sequence Cul nub a21 and Cul nub c4, express the proteins in Sf-9 insect cells and purify Cul nub a21. Also to produce polyclonal antibodies against the proteins and ligate a V5 tag into the pFastBacHTB vector to facilitate protein detection.

3 Materials and methods

- λZAP II cDNA library made from salivary glands of *Culicoides nubeculosus* (GATC Biotechnology, Germany. Provided by E.Marti, Switzerland).
- DH5α *E. coli* (Invitrogen)
- DH10Bac *E. coli* competent cells (Invitrogen)

3.1 DNA methods

Primers were designed from the a21 and c4 sequences that our coworkers in Switzerland identified with phage surface display technique. The c4 sequence found by phage display was 181 base pair and was homologous to a known sequence EU978914, primers were also designed from EU978914. Primers used in this work are listed in the appendix.

3.1.1 Amplification by PCR

PCR amplification was used to isolate the gene of interest from the λZAP II cDNA library and also to test if ligations into plasmids and bacmids had worked. The optimal DNA amount in PCR is 0.1 – 10.0 ng. PCR was performed in DNA Engine® Peltier Thermal Cycler (PTC-200) from MJ Research and Thermal Cycler 2720 from Applied Biosystems.

3.1.1.1 PCR with Taq polymerase

Taq polymerase from Fermentas and 10xThermo Buffer from New Englands BioLabs were used in this study.

PCR reaction solution	For a 20 µL PCR reaction	For a 50 µL PCR reaction
DNA	1 µl	1 – 2 µl
10x Thermo Buffer	2 µl	5 µl
2 mM dNTP mix	1 µl	2 µl
20 µM Forward primer	1 µl	2 µl
20 µM Reverse primer	1 µl	2 µl
Taq polymerase (5 units/µL)	0.1 µl	0.2 µl

dH_2O added to 20 µl or 50 µl

The reaction was performed on a thermocycler and the following program used:

PCR program

Step 1: Initial Denaturation	95^{o}C for 3 minutes
Step 2: Denature	95^{o}C for 30 sec
Step 3: Anneal primers	variable T_a-C° (ca 5° below T_m) for 20 – 40 sec
Step 4: Extend DNA	72^{o}C for 50 sec (recommended 30 sec per 500 base amplified)
Step 5: Repeat steps 2-4 for 29 cycles	
Step 6: Final Extension	72^{o}C for 7 minutes

3.1.1.2 PCR with RedTaq Ready Mix

PCR with RedTaq® Ready Mix™ from Sigma was used when bacteria cultures and bacmids were tested in PCR and most often in a 20 µl reaction. RedTaq® Ready Mix™ is a mixture of Taq polymerase, 99% pure deoxynucleotides, reaction buffer and an inert red dye in a 2x concentrate, and therefore PCR products could be loaded directly on agarose gel.

PCR reaction solution	For a 20 µL PCR reaction
DNA	1 - 2 µl
RedTaq Ready mix	10 µl
20 µM Forward primer	1 µl
20 µM Reverse primer	1 µl

ddH_2O was added until 20 µl

PCR program

Step 1: Initial Denaturation	95^oC for 3 minutes
Step 2: Denature	95^oC for 30 sec
Step 3: Anneal primers	variable T_a-C° (ca 5° below T_m) for 20 – 40 sec
Step 4: Extend DNA	72^oC for 50 sec (recommended 30 sec per 500 base amplified)
Step 5: Repeat steps 2-4 for 29 cycles	
Step 6: Final Extension	72^oC for 7 minutes

3.1.1.3 PCR with Phusion Hot Start polymerase

Phusion™Hot Start Polymerase was used in PCR to amplify the c4 gene, it is a high fidelity DNA polymerase with proof reading ability. Usually 20 µl or 50 µl reactions were made.

PCR reaction solution	For a 20 µL PCR reaction	For a 50 µL PCR reaction
DNA	1 µl	1 – 2 µl
5x HF Phusion Buffer	4 µl	8 µl
2 mM dNTP mix	2 µl	4 µl
20 µM Forward primer	1 µl	2 µl
20 µM Reverse primer	1 µl	2 µl
Phusion Hot Start polymerase (2 units/µl)	0.2 µl	0.4 µl

ddH_2O was added until 20 µl or 50 µl

PCR program

Step 1: Initial Denaturation	98^oC for 30 sec
Step 2: Denature	98^oC for 10 sec
Step 3: Anneal primers	variable T_a-C° (ca 5° below T_m) for 20 sec
Step 4: Extend DNA	72^oC for 50 sec (recommended 30 sec per 500 base amplified)
Step 5: Repeat steps 2-4 for 29 cycles	
Step 6: Final Extension	72^oC for 10 minutes

3.1.2 Agarose gel electrophoresis

The PCR amplicons were separated and visualized on a 0.8 – 1% LE agarose (Seakem®LE) gel using standard procedures. Restriction Buffer, 10x (RSB) (50%glycerol, 15 mM EDTA, 0.25% bromophenol blue) was added to the PCR amplicons made with Taq or Phusion polymerases before loading them onto the gel. TBE buffer (0.5x) (Tris borate-EDTA, 0.045 M Tris borate and 0.001 M EDTA, pH 8.0) was used as running buffer. Electrophoresis was carried out for 30 - 60 minutes at 75 Volts. To estimate the size of the bands they were compared with 1 kb ladder (Invitrogen) or 2log ladder (New Englands Biolabs). The genetic materials were visualized under UV light in White/UV Transillumator (UVP, UK) and photographed with the Grab-It program (UVP, UK) and finally printed out with Sony Digital Graphic Printer (UP-D860E).

3.1.3 DNA quantification

DNA samples were quantified in NanoDrop®ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.) as described in the manual of the manufacturer.

3.1.4 Restriction digest and cloning

A BamHI restriction site was incorporated on the 5´-end af the forward primers and a HindIII/XhoI site on the 5'-end af the reverse primers to enable cloning of the coding sequence into pFastbac HT vector (Invitrogen). The coding sequence with restriction sites was amplified by PCR and run on a 1% LB agarose gel. Then the band was excised under UV light and isolated on a Qiagen Spin Column according to the manufacturer´s protocol (Qiagen).

The endonucleases BamHI and HindIII/XhoI were used to double digest the PCR amplicon and the vector according to the manufacturer´s protocol (Fermentas). The insert was then ligated into the vector at the respective sites with T4 DNA ligase from Fermentas over night as described by the manufacturer.

3.1.5 Sequencing

Sequencing was done using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the ABI310 according to manufacturer´s protocol. Corresponding vector primers were used to sequence the vector clones of interest, but also primers designed from the given gene sequences (a21 and c4) to get high quality sequence of the whole gene in the vector.

3.1.6 DNA and protein analysis

Data from sequencing were analyzed in Sequencher 4.6 - 4.8 to create a contiguous consensus sequence. The DNA and protein sequences were identified by using analyzing tools on the website of National Centre for Biotechnology (NCBI). Tools on Expert Protein Analyzes System (EXPASY) hosted by the Switzerland institute of Bioinformatics were used for further protein sequence analyzes. For sequence alignment CLUSTAL 2.0.12 multiple sequence alignment tool was used from the website of the European Bioinformatics Institude (http://www.ebi.ac.uk).

3.2 Expression of recombinant Cul nub proteins with the baculovirus system in Sf-9 insect cells

The Bac-to-Bac® Baculovirus System from Invitrogen is a fast and efficient method to generate recombinant baculoviruses. It is based on site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) raised in *E. coli*.

3.2.1 Transformation and selection of recombinant DH10Bac cells

The pFastBac HT B™ expression cassette is flanked by the left and right arms of the site specific Tn7 transposon and contains a polyhedrin promoter, a multiple cloning site, a gentamicin resistance gene and an SV40 polyadenylation signal forming a mini-Tn7.

The MAX Efficiency® chemically competent DH10Bac™ *E. coli* strain used contains a target bacmid and a helper plasmid. The target bacmid is a shuttle vector containing the 136 kb baculovirus genome, with a mini-*att*Tn7 target site, a kanamycin resistance gene, a LacZα gene that provides confirmation of transposition at the correct site (gives Lac^- phenotype) and a mini-F replicon which allows stable replication in *E. coli*. The helper plasmid encodes the transposase gene and a tetracycline resistance gene.

The pFastBac HT B™ construct was transformed by heatshock into the competent DH10Bac™ *E. coli* (Invitrogen) for transposition at the mini-*att*Tn7 site of the bacmid. After transformation the cells were incubated in SOC broth for 4 hours before plating out on LB-agar containing 50 µg/ml kanamycin, 7 µg/ml gentamycin, 10 µg/ml tetracyclin, 200 µg/ml X-gal and 30 µl IPTG (100mM). Blue/white selection was used to pick white Lac^- recombinant bacmid colonies and inoculated in LB-medium with the appropriate antibiotics and grown until stationary phase (48 hours). Bacteria cultures were then tested in PCR with M13 primers and primers specific for the gene and cultures containing recombinant bacmids were then purified and used in transfection of Sf-9 insect cells. All methods were according to the Bac-to-Bac manual (Invitrogen).

3.2.2 Sf-9 insect cells

Sf-9 cells were maintained in SF-900II medium (Invitrogen) supplemented with 100 IU penicillin, 100 IU streptomycin (pen/strep) and 1% fetal bovine serum (FBS). They were seeded 0.3-0.8 $x10^6$/ml and thrived well up to 2.5$x10^6$/ml cell density. Virus production was done in SF-900II with 1% FBS but protein production in SF-900II without FBS. All maintainance and incubation of Sf-9 cells was done in 27°C closed culture.

3.2.3 Transfection into Sf-9 cells and passage of virus

Transfection with Cellfectin (Invitrogen) and Lipofectamine 2000 (Invitrogen) was compared. The day before transfection Sf-9 cells in good condition were seeded 0,3$x10^6$ cells/well into a 12 well plate (Nunc) in Sf-900II medium without antibiotics and serum, 1.0 ml cell solution per well.

Transfection with cellfectin:

Transfection was done according to the Bac-To-Bac manual from Invitrogen. Graces medium unsupplemented was used throughout the procedure. On the day of transfection, cellfectin was mixed

carefully and 8.0 µl added into 100.0 µl Grace´s medium. Different amounts of the bacmid, 1.5, 3 and 6 µg were added into 100 µl Grace´s medium and mixed gently (recommended 1-2 µg in the Invitrogen protocol). The mixtures containing both cellfectin and bacmid were mixed gently together and incubated for 15-45 minutes at RT. Cellfectin in Grace´s medium was used as control. Meanwhile, the medium was removed from each well and the cells rinsed once with 1.0 ml Grace´s medium. Grace´s medium, 0.8 ml, was added to each bacmid-cellfectin mixture and the control, mixed gently and then added to the wells. The cells were incubated for 5 hours. Then the bacmid-cellfectin mixture was removed from each well and 1.0 ml Sf-900II medium with pen/strep and 1% FBS was added. The plate was then incubated for 72 hours or until first sign of viral infection was seen.

After 4-6 days 0.3 ml supernatant from the wells was transferred into a new plate with fresh 1.0 ml Sf-9 cells. After 10-12 days the cells were tested in WB.

Transfection with Lipofectamine:

The day of transfection**,** lipofectamine 2000 (Invitrogen) was vortexed and 3.5 µl added into Opti-mem (Invitrogen) to a total of 85 µl. Incubated for 5 minutes at RT**.** Different amounts of the bacmid, 1.5, 3.0 and 6.0 µg were added into Opti-mem medium to a total of 85 µl (recommended 1-2 µg in the Invitrogen protocol). The lipofectamine mixture was added into the bacmid mixture 30 min, and incubated for 20 min at RT. Lipofectamine in Opti-mem medium was used as control.

Medium, 0.5 ml, was removed from each well, 0.5 ml remained. The transfection mixture and control mixtures added to the wells were incubated for 5 hrs. Then 1.5 ml Sf-900II medium with 1% FBS and pen/strep was added.

The plate was then incubated for 9-11 days under careful observation and comparison with controls until first sign of viral infection was seen.

To analyse the transfections and passages in WB each well was harvested and spun at 10,000 rpm for 7 minutes and the supernatant discarded. The pellet was resuspended in 30 µl phosphate buffered saline (PBS) and diluted 1:1 in 2x reducing buffer.

3.2.4 Amplification of virus stock

To obtain high titer virus stock several passages were done. Cellfectin transfection cultures were passaged after 4-6 days but the lipofectamine cultures after 9-11 days. Whole cell suspension were passaged with lipofectamine cultures after transfection but in all other passages 0.3-0.5 ml of supernatant was transferred into 1 ml of fresh Sf-9 cells.

At least 4 passages of each virus stock in fresh Sf-9 cells was done to obtain higher titer virus stock. Virus stocks were kept at 4°C for over 3 months and frozen at -80°C for long time storage.

3.2.5 Titration of recombinant baculovirus

Sf-9 cells were seeded in a 96 well flat bottom plate (Nunc), $1x10^5$ cells/ml (100 µl per/well), the day before. Seven tenfold dilutions of the viral stock 10^{-1}-10^{-7} were tested in Sf-900II medium with 1% FBS, and each dilution added in 100 µl to 4 wells and as a control 100 µl of medium alone was added into the remaining wells. The plate was incubated and examined for cytopathy in a microscope daily for 14 days.

3.2.6 Time kinetics of recombinant protein production

Optimal harvest time was decided for each protein. Four T25 flasks (Nunc) with confluent Sf-9 cells were infected with 0.5 ml recombinant virus. Control flask contained only medium. The flasks were kept on a rocking platform for one hour at RT. Then 4.0 ml of Sf-900II medium without FBS was added to each flask and incubated at 27°C. Cells were harvested at different time points; day 4, 6, 8 and 10, negative control was harvested at day 8.

Cells were harvested by pumping them off the plastic and spun at 2000 rpm for 12 minutes at 10°C. The supernatant was discarded and the pellet resuspended in a sterile lysis buffer (1% Triton-X-100 in PBS), supplemented with 200 units/ml of DNase I (Fermentas), the first harvest was also supplemented with protease inhibitor cocktail (Sigma) 60 µl per 1 ml. Pellet from T25 flask was solved in 200 µl and from 5 large petri dishes in 3 ml. The resuspended pellet was incubated on a shaker for 30 minutes at RT, then transferred to eppendorf tubes and centrifuged at 10,000 rpm for 7 minutes. The supernatant was aliquoted into cryo-vials and the pellet resuspended in lysis buffer, same volume as before and aliquoted into cryo-vials. The aliquots were kept at -80°C until purification. Aliquots were also added into 2x reducing buffer for WB analysis.

3.2.7 Protein production with the baculovirus system in Sf-9 cells

Sf-9 cells in exponential growth on large petri dishes, 145 cm^2 (Nunc), at 2.0-3.0x10^6 cells/ml in 27 ml of Sf-900II serum free medium were infected with 3 ml of high titer recombinant virus. Harvested at the day decided by titration as described in 3.2.6

3.3 Protein methods

3.3.1 SDS Polyacrylamide electrophoresis

The proteins were separated by SDS-PAGE in the Mini-protean II system (Bio-Rad) by the originally described method by Laemmli 1970 (Laemmli, 1970). The samples were denatured with 2x sample buffer (0,5% 2-mercaptoethanol + 20% glycerol + 2% SDS in 0.5 M Tris), boiled at 100°C for 5 minutes, spun down at 3000 rpm for 3 minutes and run on 12% polyacrylamide gels under reducing conditions. To estimate the size of the proteins prestained molecular mass marker (Fermentas, #SM0671) was used. The proteins were visualized using Western blotting on PVDF membranes (Millipore) or Coomassie blue staining.

3.3.2 Western Blot

After SDS-Page the proteins were transferred to a PVDF membrane (Millipore) by wet transfer in the Mini-protean II system (Bio-Rad). The PVDF membrane was prepared by pre-wetting it in methanol for 15 sec., water for 2 minutes and finally transfer buffer (25 mM Tris, 192 mM Glycine, 20 % methanol) for 5 minutes. Filter papers, pads and gel were equilibrated in transfer buffer. The transfer sandwich was made in transfer buffer and transfer was done at 100 Volt, 250 mA for 1 hour at 4°C. After the transfer the membranes were blocked with TBS-T (tris buffered saline containing 0.1% Tween) with an extra 2% Tween, for 30 minutes. The membranes were incubated over night at 4°C with a primary antibody, polyclonal mouse anti-6xHis (Santa Cruz Biotechnology), monoclonal mouse anti-V5

(Invitrogen) or polyclonal mouse ascites made as described in 3.4, followed by a secondary antibody, phosphatase labelled goat anti-mouse (Dako) (1:5000). In between steps the membranes were washed 5 times with TBS-T at RT. The membranes were developed using BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazoliumchloride) (Roche) in alkaline phosphatase (100mM Tris-HCl, 100mM NaCl, 5mM $MgCl_2$, 0.05% Tween 20, pH 9.5) in dilution 1:50.

3.3.3 Coomassie blue staining

The washing and the elution fractions from the protein purification were run at 12% SDS-PAGE gel and then Coomassie blue stained as described by Wong et al 2000 ((Wong et al., 2000).

3.3.4 Protein purification

Cul-nub a21 and cul-nub a21/V5 proteins were purified with His-select Nickel Affinity gel from Sigma (H0537). Because the proteins are found in inclusion bodies they were first resuspended in Lysis buffer A (6M Guanidine-HCl, 0.1M $NaH_2PO_4xH_2O$, pH 8.0), 0.5 ml Sf-9 cell pellet in 5 ml lysis buffer A, and sonicated 5 times (10 sec pulse + 20 sec pause) on ice (denature condition).

1. The gel was washed and equilibrated as following: 0.5 ml gel in 50 ml tube, washed with 5 ml ddH_2O (10x gel volume), centrifuged (3000 rpm, 5 minutes, RT) and supernatant discarded, washed again with lysis buffer A as with ddH_2O.
2. The equilibrated gel mixed with the cell lysate on an orbital shaker (30 minutes, RT), centrifuged (3000 rpm, 5 minutes), supernatant harvested (SN#1) for analysis.
3. Gel resuspended in 5 ml Wash buffer B (8M Urea, 0.1M $NaH_2PO_4xH_2O$, 0.01M Tris base, 0.1M NH_4Cl, pH 8.0), mixed for 5 minutes and then centrifuged (3000 rpm, 5 minutes), supernatant harvested (SN#2).
4. Gel resuspended in 5 ml Wash buffer C (same composition as buffer B, pH 6.3), mixed for 5 minutes and then centrifuged (3000 rpm, 5 minutes), supernatant harvested (SN#3).
5. Elution buffer E1 (same composition as buffer B, pH 4.4), 1 ml, added to the gel, mixed for 10 minutes, transferred onto column with 0.2 µm membrane, eluate collected (E1#1).
6. Elution repeated with buffer E1 and eluate collected (E1#2) and then repeated twice with elution buffer E2 (same composition as buffer B, pH 4.0) and eluates collected (E2#1 and E2#2).
7. The gel washed first with 1 ml buffer F (6M Guanidine-HCl, 0.2M acetic acid) then 1 ml ddH_2O and finally with 30% ethanol.

The wash samples (SN#2 and SN#3) and elution samples (E1#1-2 and E2#1-2) were tested by SDS-PAGE followed by coomassie blue staining and WB.

3.3.5 Deglycolysation

A PNGase kit from New England Biolabs was used for deglycosylation of cul-nub-a21 and cul-nub-a21/V5 following the manufacture´s protocol.

3.4 Polyclonal antibody production

Production of polyclonal antibodies against candidate allergens was in mouse ascetic fluid as described by Overkamp et al. 1988 (Overkamp et al., 1988). Recombinant purified *C. nubeculosus* proteins (expressed in *E. coli*) were mixed in Complete Freund´s adjuvant (CFA) at a protein concentration 20 µg per mouse. Three BALB/c mice for each protein were injected intraperitoneally (i.p.). The second injection was done on day 14 with the proteins in Incomplete Freund´s adjuvant (IFA) and the mice also received 500 µl of Pristane (Sigma). The third i.p. injection was done at day 21 using protein in IFA. The following days the mice were observed, sacrificed and the ascitis was collected, aliqouted and stored at -20°C.

4 Results

4.1 V5 Tag insertion into pFastBac HT B

To facilitate protein detection the V5 tag was PCR amplified from pcDNA3.1/V5-His B vector (Invitrogen) with primers that had BamHI and HindIII restriction sites, the V5 tag is 196 base pairs including the primers (fig 7). The V5 tag insert includes a 6xHis, the FastBac HT B – V5 has tag therefore two tags of 6xHis.

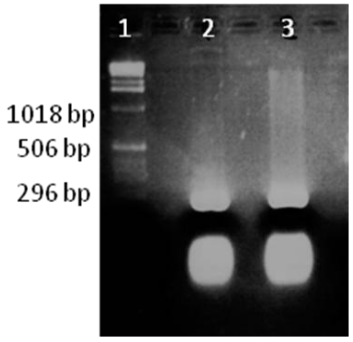


Figure 7: PCR amplification of V5 tag with V5-BamHI fw and V5-HindIII rev primers. Lane 1. Ladder, **lane 2-3.** V5 Tag from pcDNA3.1/V5-His B.

The product was isolated and cut with the restriction enzymes BamHI and HindIII and ligated into the Fastbac HT B vector (fig 8). The presence of the V5 sequence in Fastbac vector was confirmed by PCR (fig 9.), the size of the product is 301 base pairs.

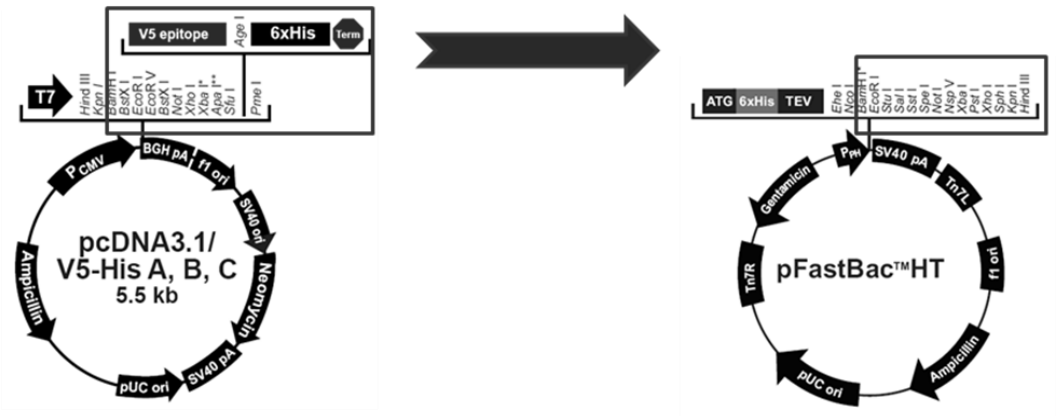


Figure 8: Schematic picture of insertion of the V5 tag into the pFastBac HT B vector. The part of pcDNA3.1/V5-His B marked by a box was ligated into the pFastbac HT B vector.

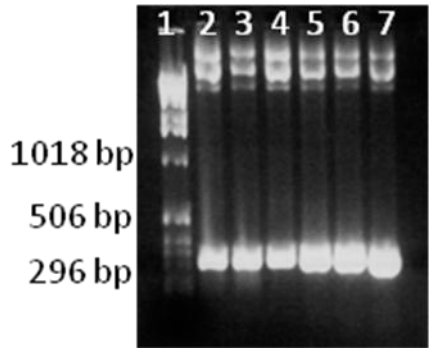


Figure 9: PCR of pFastBac HT B – V5 vector with Fastbac fw and V5-HindIII rev primers. Lane 1. Ladder, **lane 2-7.** V5 Tag in Fastbac HT B.

Sequencing FastBac HT B – V5 showed that the V5 tag was in correct phase when translated into amino acids (fig 10).

A

```
1  ATGTCGTACTACCATCACCATCACCATCACGATTACGATATCCCAACGACCGAAAACCTG 60
2  ATGTCGTACTACCATCACCATCACCATCACGATTACGATATCCCAACGACCGAAAACCTG 60
   ************************************************************

                       BamHI
1  TATTTTCAGGGCGCCATGGGATCCACTAGTCCAGTGTGGTGGAATTCTGCAGATATCCAG 120
2  TATTTTCAGGGCGCCATGGGATCCACTAGTCCAGTGTGGTGGAATTCTGCAGATATCCAG 120
   ************************************************************

1  CACAGTGGCGGCCGCTCGAGTCTAGAGGGCCCGCGGTTCGAAGGTAAGCCTATCCCTAAC 180
2  CACAGTGGCGGCCGCTCGAGTCTAGAGGGCCCGCGGTTCGAAGGTAAGCCTATCCCTAAC 180
   ************************************************************

1  CCTCTCCTCGGTCTCGATTCTACGCGTACCGGTCATCATCACCATCACCATTGAGTTTAA 240
2  CCTCTCCTCGGTCTCGATTCTACGCGTACCGGTCATCATCACCATCACCATTGAGTTTAA 240
   ************************************************************

             HindIII
1  ACCCGCTGACGAAGCTTGTCGAGAAGTACTAGAGGATCATAATCAGCCATACCA------ 294
2  ACCCGCTGACGAAGCTTGTCGAGAAGTACTAGAGGATCATAATCAGCCATACCACATTTG 300
   ************************************************************
```

B

```
                                                          V5-Tag
1  MSYYHHHHHHDYDIPTTENLYFQGAMGSTSPVWWNSADIQHSGGRSSLEGPRFEGKPIPN 60
2  MSYYHHHHHHDYDIPTTENLYFQGAMGSTSPVWWNSADIQHSGGRSSLEGPRFEGKPIPN 60
   ************************************************************

1  PLLGLDSTRTGHHHHHH-V-TR-RSLSRSTRGS-SAIP-- 94
2  PLLGLDSTRTGHHHHHH-V-TR-RSLSRSTRGS-SAIPHL 96
   ****************** * ** ********** ****
```

Figure 10: The sequence of Fastbac HT B – V5 and FastBac HT B (Invitrogen). A. Nucleotide sequence, **B.** Amino acid sequence.

4.2 Cul nub a21

4.2.1 Isolation of the Cul nub a21 gene

Primers with restriction sites were designed after a given sequence, 1215 base pair (405 amino acids) till the first stop codon, from our coworkers in Switzerland and the Cul nub a21 gene was isolated with PCR from λ ZAP II cDNA salivary gland library of *C. nubeculosus* (fig 11).

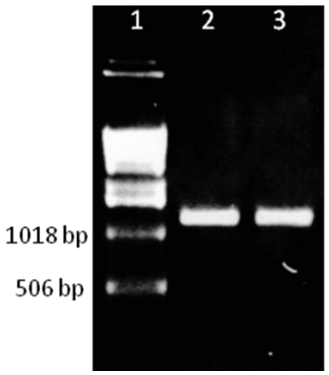


Figure 11: PCR of Cul nub a21 gene with cnpen-BamHI fw and cnpen-XhoI rev primers. Lane 1. Ladder, lane 2-3. Cul nub a21.

4.2.2 Cloning of Cul nub a21 into pFastBac HT B and pFastBac HT B-V5

The cul nub a21 amplicon was ligated into Fastbac HT B and Fastbac HT B – V5 at the restriction sites BamHI and XhoI and sequenced to confirm the correct phase.

Sequencing gave a 1215 base pair Cul nub a21 gene, or 405 amino acids (without the stop codon). When translated into amino acids it was in phase with the Fastbac HT B and the Fastbac HT B – V5 (see appendix).

An alignment search for the Cul nub a21 (1215 base pair) sequence with blast*n* (NCBI) showed 99% identity to the *Culicoides nubeculosus* Cul n 5 allergen and 83% identity to the nucleotide sequence AY603639, an unknown salivary protein from *Culicoides sonorensis*. A protein blast gave 99% identity to Cul n 5 protein from *Culicoides nubeculosus*.

4.2.3 Cul nub a21 and Cul nub a21 – V5 bacmids

Fastbac HT B and Fastbac HT B – V5 vectors and the construct, Cul nub a21, were transformed into DH10Bac *E. coli*. Recombinant DH10Bac colonies were analysed for Cul nub a21 in PCR. Two separate bacmid preperations of rCul nub a21 and five of rCul nub a21-V5 were made and transfected into Sf-9 cells in different DNA concentrations with Cellfectin and lipofectamine. To ascertain that the genes went in the bacmid they were analysed in PCR with Cul nub a21 specific primer and M13 primer specific for the bacmid (fig 12).

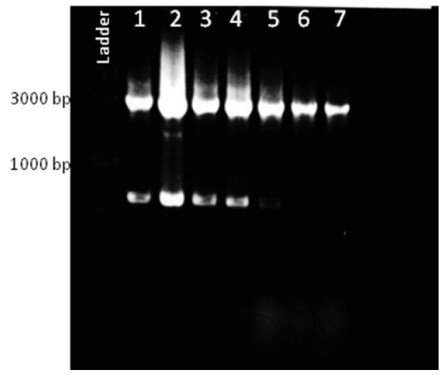


Figure 12: PCR of Cul nub a21 and Cul nub a21-V5 bacmids. Lane 1.-2. Cul nub a21 in bacmid, **3.-7.** Cul nub a21-V5 in bacmid.

4.2.4 Transfection with cellfectin and lipofectamine

There was some difference in the cytopathy between cellfectin transfection and lipofectamine transfection, the cellfectin transfection showed earlier cytopathy than the lipofectamine. Cytopathy was seen in one Cul nub a21 Bac-1 1.5 µg DNA in both transfection with cellfectin and lipofectamine. When tested in WB the cellfectin sample came positive in the transfection but the lipofectamine was not positive until in the first passage.

Transfections were done with two Cul nub a21 bacmids in cellfectin and lipofectamine, Cul nub a21 bac-1 and Cul nub a21 bac-2, and three Cul nub a21–V5 bacmids in cellfectin, Cul nub a21-V5 bac-4, Cul nub a21-V5 bac-5 and Cul nub a21-V5 bac-6 (Table 1).

Table 1: Cul nub a21 bacmids used in transfection

Bacmid	*DNA concentration*	*Cytopathy*	*WB*
Cul nub a21 Bac-1	**1.5 µg**	**+**	**+**
	3.0 µg	-	-
	6.0 µg	-	-
Cul nub a21 Bac-2	1.5 µg	-	-
	3.0 µg	-	-
	6.0 µg	-	-
Cul nub a21-V5 Bac-4	1.5 µg	-	-
	3.0 µg	**+**	**+**
	6.0 µg	**+**	**+**
Cul nub a21-V5 Bac-5	**1.5 µg**	**+**	**+**
	3.0 µg	-	-
	6.0 µg	-	-
Cul nub a21-V5 Bac-6	1.5 µg	-	-
	3.0 µg	**+**	**+**
	6.0 µg	-	-

Normal Sf-9 cells are round and regular, whereas upon infection they are large and irregular giant cells with a different light refraction but after passages they become irregular and more oval (fig. 13).

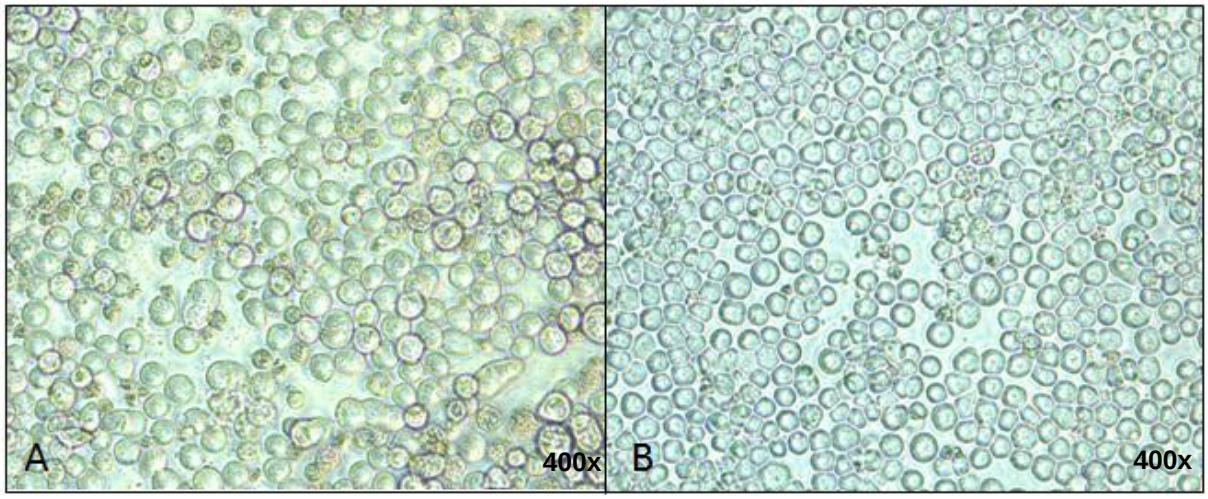


Figure 13: Recombinant baculovirus in Sf-9 cells seen in a light microscope. A. Baculovirus infected Sf-9 cells, **B.** Healthy control Sf-9 cells.

4.2.5 Expression of recombinant Cul nub a21 and Cul nub a21-V5 in Sf-9 cells shown in WB

In WB the polyclonal anti Cul nub a21 antibody bound specifically to ~53 and ~43 kDa protein bands in rCul nub a21 and ~55 and ~45 kDa protein bands in rCul nub a21-V5. According to the open reading frame of the Cul nub a21 insert with the 6xHis-tag upstream from the vector, the expected size should be ~50 kDa, and the cul nub a21-V5 insert with the double 6xHis-tag (both upstream and downstream) and the V5-tag from the vector should be ~52 kDa. Expression of rCul nub a21 after transfection with cellfectin was detected in two samples, both with 1.5 µg DNA concentrations in Cul nub a21 bac-1, and in one sample with lipofectamine after first passage, Cul nub a21 bac-1 with 1.5 µg DNA conentration (fig. 14).

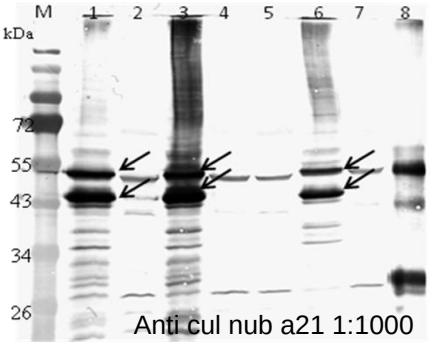


Figure 14: WB of rCul nub a21 after first passage. Lane **1.-5.** are with cellfectin, lane **6.-7.** are with Lipofectamine. Lane **1.** Cul nub a21 bac-1 1.5 µg, **2.** Cellfectin control, **3.** Cul nub a21 bac-1 1.5 µg, **4.** Cul nub a21 bac-2 1.5 µg, **5.** Cellfectin control, **6.** Cul nub a21 bac-1 1.5 µg **7.** Lipofectamine control, **8.** *E. coli* produced Cul nub a21.

The expression of rCul nub a21-V5 was detected in four samples, with 3.0 and 6.0 µg DNA concentrations in Cul nub a21-V5 bac-4, 1.5 µg DNA concentrations in Cul nub a21-V5 bac-5 and in 3.0 µg DNA concentration Cul nub a21-V5 bac-6 (fig. 15).

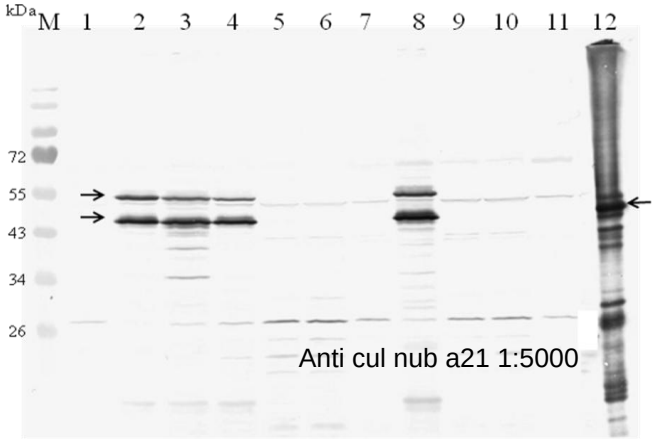


Figure 15: WB of rCul nub a21-V5 after transfection. Lane 1.-3. Cul nub a21-V5 bac-1, 1.5 µg, 3.0 µg, 6.0 µg. **4.-6.** Cul nub a21-V5 bac-2, **7.-9.** Cul nub a21-V5 bac-3, **10.** Sf-9 Cell control . **11.** Cellfectin control. **12.** *E. coli* produced Cul nub a21.

The V5 tag was shown to be expressed by comparing binding of rCul nub a21 – V5 with the polyclonal anti a21 antibody and with the monoclonal anti V5 antibody. The anti V5 antibody bound to bands of the same size as the anti a21 antibody (fig. 16).

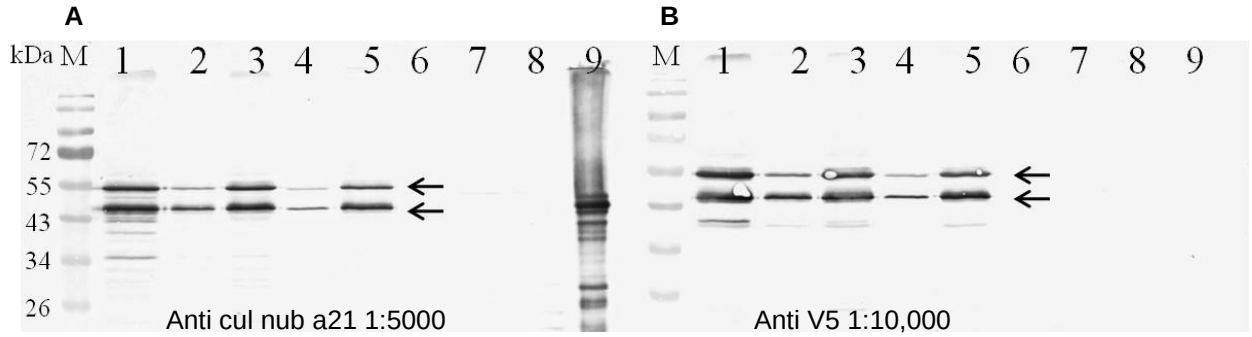


Figure 16: Comparison between polyclonal anti a21 (A) and monoclonal anti V5 (B). Lane 1.-2. rCul nub a21–V5 Bac-1, 3.0 µg and 6.0 µg DNA, **3.-4.** rCul nub a21–V5 Bac-2, 1.5 µg and 3.0 µg DNA, **5.-6.** rCul nub a21–V5 Bac-3, 3.0 µg and 6.0 µg DNA, **7.** Sf-9 Cell control, **8.** Cellfectin control, **9.** *E. coli* produced Cul nub a21.

The pellets and supernatants from lysed infected Sf-9 cells harvested at day 2-3 were tested in WB. rCul nub a21 was found in the pellet (fig. 17).

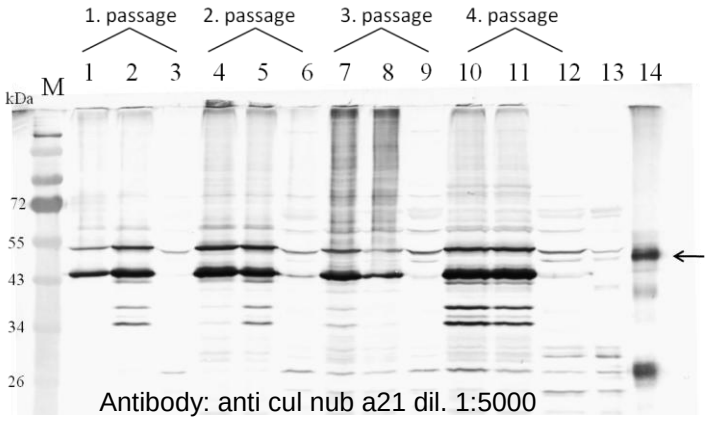


Figure 17: WB of rCul nub a21 expressed in Sf-9 cells. Passage 1-4: lane **1.-2.** pellet, dil. 1:8, 1:10, **3.** Supernatant, **13.** Cellfectin control, **14.** *E. coli* produced Cul nub a21.

4.2.6 Titration of rCul nub a21 baculovirus

Titration was done in Sf-9 cells to establish optimal time point for harvesting infectious virus. Virus was harvested at days 2, 3 and 4 and titer determined assessing cytopathy in a light microscope. Day 3 proved to be the optimal day for harvesting for further infection for rCul nub a21. At that time point the titer was 10^7 TCID/ml.

4.2.7 Time kinetics for harvesting of recombinant protein

Sf-9 cells were harvested at days 4, 6, 8 and 10. The optimal time point for protein harvesting was on day 4 and 6 for rCul nub a21 (fig. 18).

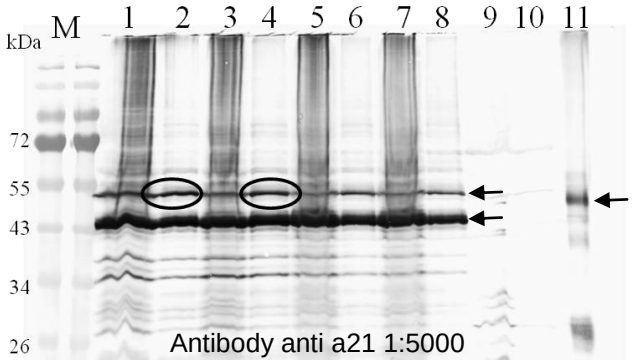


Figure 18: Optimal time point for harvesting of rCul nub a21. Odd numbers are dil. 1:1, even numbers are dil. 1:20. Lane 1.-2. Day 4, **3.-4.** Day 6, **5.-6.** Day 8, **7.-8.** Day 10, **9.-10.** Sf-9 cell control harv. Day 8, **11.** *E. coli* produced rCul nub a21.

4.2.8 Cul nub a21 and Cul nub a21-V5 protein purification

rCul nub a21 and rCul nub a21-V5 proteins were purified with His-select Nickel Affinity gel. Because the proteins were found in inclusion bodies (insoluble aggregates) in the Sf-9 cell the proteins had to be purified in a denatured form. The washing fractions and the elution fractions were run on SDS-PAGE and analysed by Coomassie staining (fig. 19) and WB (fig. 20).

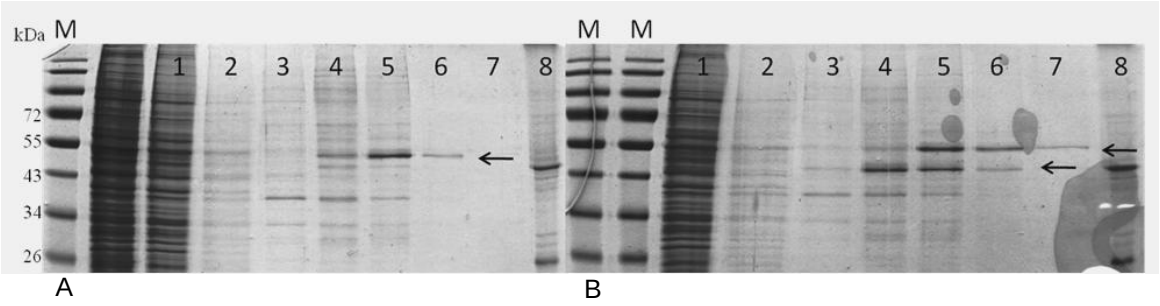


Figure 19: Coomassie blue staining of His-select Nickel affinity gel purification. A. Purification of rCul nub a21 and B. Purification of rCul nub a21-V5. Lane **1.** rCul nub extract, **2.- 3.** Washing fractions, **4.-7.** Eluted fractions, **8.** *E. coli* produced rCul nub a21.

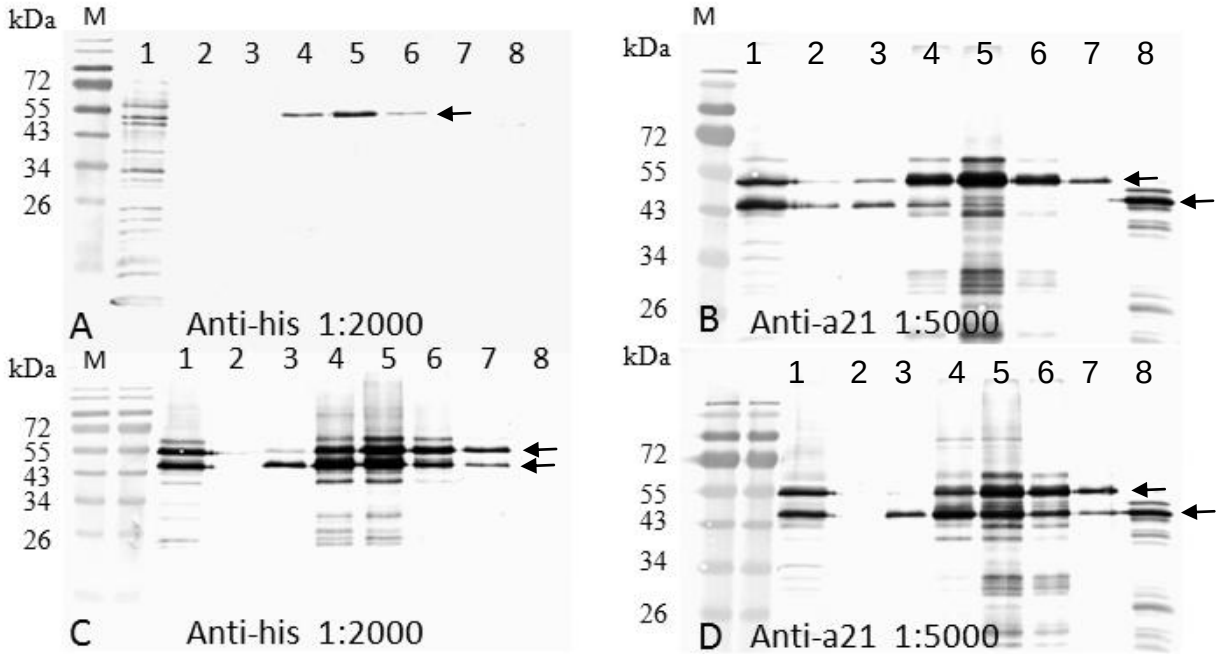
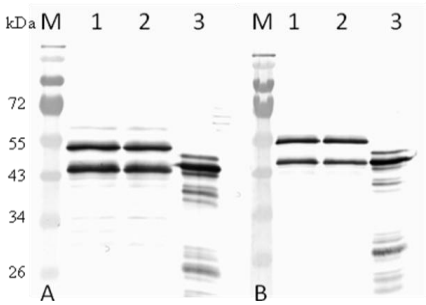


Figure 20: WB analysis of His-select Nickel affinity gel purification of rCul nub a21. A and B: rCul nub a21, **C and D: rCul nub a21 – V5. Lane 1.** Unpurified recombinant protein, **2.-3.** Washing fractions, **4.-7.** Eluted fractions, **8.** *E. coli* produced rCul nub a21.

Figure 20 also shows that the anti 6xHis antibody is binding more strongly to rCul nub a21 – V5 than to rCul nub a21 without the V5 tag.

4.2.9 Deglycosylation of Cul nub a21 and Cul nub a21-V5

The rCul nub a21 and rCul nub a21 – V5 proteins were treated with PNGase F, an enzyme that cleaves intact N-linked glycans from glycoproteins. WB analyses show no difference in the sizes of the protein bands indicating that they are not glycoproteins but this was only tried once and needs further testing (fig. 21).



Anti cul nub a21 1:5000

Figure 21: PNGase F treatment of rCul nub a21 (A) and rCul nub a21 – V5 (B) samples vs. untreated samples. Lane 1. Untreated rCul nub a21, **2.** PNGase F treated rCul nub a21, **3.** *E. coli* produced rCul nub a21.

4.3 Cul nub c4

4.3.1 Isolation of the Cul nub c4 gene

Primers with restriction sites were designed after the published sequence EU978914 and used to isolate the Cul nub c4 gene from the λ ZAP II cDNA salivary gland library of *C. nubeculosus*. The Cul nub c4 gene is 1254 base pairs and codes for 418 amino acids (fig. 22).

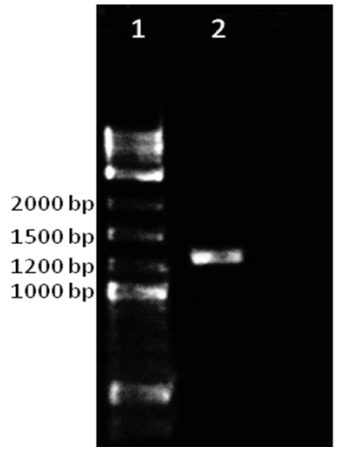


Figure 22: PCR of Cul nub c4 gene with CNSG79-BamHI fw and CNSG79-XhoI rev primers. Lane 1. 2log Ladder, **2.**Cul nub c4 gene.

4.3.2 Cloning of Cul nub c4 into pFastBac HT B and pFastBac HT B-V5

The cul nub c4 amplicon was ligated into Fastbac HT B and Fastbac HT B – V5 at the restriction sites BamHI and XhoI and sequenced to confirm the correct phase.

Sequencing gave a 1254 base pair Cul nub c4 gene, or 418 amino acids (without the stop codon). When translated into amino acids it was in phase with the Fastbac HT B and the Fastbac HT B – V5 (see appendix). An alignment search for the Cul nub c4 (1252 base pair) sequence with blast*n* (NCBI) showed 97% identity with *Culicoides nubeculosus* clone CNSG79 secreted salivary protein mRNA (EU978914) and 96% identity with Culicoides nubeculosus Cul n 10 allergen mRNA (HM145958). A protein blast gave 98% identity (99% positives) with secreted salivary protein from *Culiciodes nubeculosus* (ACM40903) and 94% identity (97% positives) with the Cul n 10 allergen from *Culicoides nubeculosus* (ADJ67274).

4.3.3 Cul nub c4 and Cul nub c4 – V5 bacmids

Fastbac HT B and Fastbac HT B – V5 vectors and the construct were transformed into DH10Bac *E. coli*. Recombinant DH10Bac colonies were analysed for Cul nub c4 in PCR. Four separate bacmid preperations of rCul nub c4 and three of rCul nub c4-V5 were made and transfected into Sf-9 cells in different DNA concentrations with Cellfectin and lipofectamine. To ascertain that the gene was in the bacmid they were analysed in PCR with Cul nub c4 specific primer with M13 primer specific for the bacmid (fig 23).

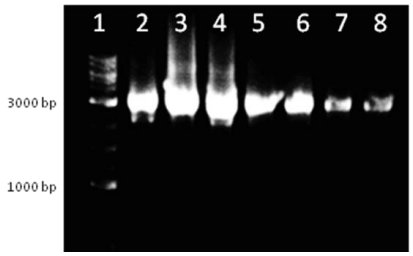


Figure 23: PCR of Cul nub c4 and Cul nub c4-V5 bacmids. Lane 1. 2log ladder, **2-5.** Cul nub c4, **6.-8.** Cul nub c4-V5.

4.3.4 Expression of recombinant Cul nub c4 and Cul nub c4-V5 in Sf-9 cells

Transfections were done with two Cul nub c4 bacmids in cellfectin and lipofectamine, Cul nub c4 Bac-1 and Cul nub c4 Bac-2, and three Cul nub c4 – V5 bacmids in cellfectin, Cul nub c4 – V5 Bac-1, Cul nub c4 – V5 Bac-2 and Cul nub c4 – V5 Bac-3.

There was detectable cytopathy in one sample of Cul nub c4 transfection with lipofectamine, Cul nub c4 Bac-1, 1.5 µg DNA, and in two samples with Cul nub c4 – V5 transfection with cellfectin, 1.5 µg DNA in Cul nub c4 – V5 Bac-1 and 3.0 µg DNA in Cul nub c4 – V5 Bac-3 (Table 2).

Table 2: Cul nub c4 bacmids used in transfection

Bacmid	*DNA concentration*	*Cytopathy*	*WB*
Cul nub c4 Bac-1	**1.5 µg**	**+**	-
	3.0 µg	-	-
	6.0 µg	-	-
Cul nub c4 Bac-2	1.5 µg	-	-
	3.0 µg	-	-
	6.0 µg	-	-
Cul nub c4-V5 Bac-1	1.5 µg	**+**	**+**
	3.0 µg	-	-
	6.0 µg	-	-
Cul nub c4-V5 Bac-2	1.5 µg	-	-
	3.0 µg	-	-
	6.0 µg	-	-
Cul nub c4-V5 Bac-3	1.5 µg	-	-
	3.0 µg	**+**	**+**
	6.0 µg	-	-

The polyclonal anti c4 did not bind specifically to any protein bands in WB. The rCul nub c4 – V5 samples were analysed again in WB with monoclonal anti V5 antibody and polyclonal anti c4 antibody for a comparison, showing that the anti c4 antibody is not working, because the anti V5 antibody binds to ~57 kDa and ~47 kDa protein bands. The expected size of rCul nub c4 – V5 should be ~54 kDa (fig. 24).

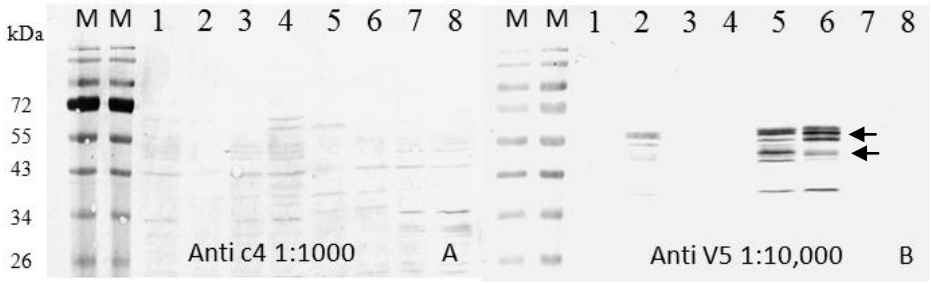


Figure 24: Comparison between polyclonal anti c4 (A) and monoclonal anti V5 (B). Lane 1-3: transfection, lane 4-8: 1.passage. 1. Cul nub c4-V5 Bac-1, 1.5 µg DNA, **2.** Cul nub c4-V5 Bac-1, 3.0 µg DNA, **3.** Cellfectin control, **4.** Cellfectin control, **5.** Cul nub c4-V5 Bac-1, 1.5 µg DNA, **6.** Cul nub c4-V5 Bac-3, 3.0 µ DNA, **7.** Cul nub c4-V5 Bac-2, 1.5 µ DNA, **8.** Cul nub c4-V5 Bac-3, 6.0 µg DNA

Since the anti c4 did not bind further test were performed with the rCul nub c4 –V5 samples.

The pellets and supernatants from lysed infected Sf-9 cells were tested for protein expression in WB. rCul nub c4 – V5 was found in the pellet (fig. 25).

4.3.5 Titration of rCul nub c4-V5 baculovirus

Titration was done in Sf-9 cells to establish optimal time point for harvesting infectious virus. Virus was harvested at day 2, 3 and 4 and titer determined assessing cytopathy in a light microscope. Day 3 proved to be the optimal day for harvesting virus for further infection. At that time point the titer was very low, 10^3 TCID/ml.

4.3.6 Time kinetics for harvesting recombinant protein

Sf-9 cells were harvested at days 4, 6, 8 and 10 after infection with rCul nub c4-V5. The optimal time point for protein harvesting was on day 8 for rCul nub c4 – V5 (fig. 25).

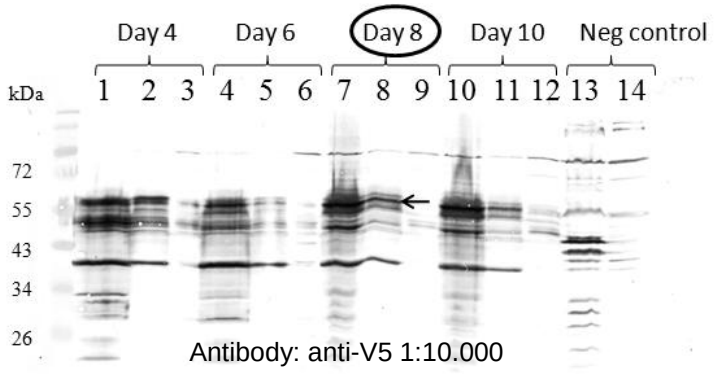


Figure 25: Optimal time point for harvesting of rCul nub c4 – V5. Lane 1,4,7,10,13: pellet dil. 1:1, **lane 2,5,8,11:** pellet 1:20, **lane 3,6,9,12,14:** supernatant. Neg. control: Sf-9 cells.

5 Discussion

Insect bite hypersensitivity (IBH) is a severe problem in exported Icelandic horses. About 50% of them get IBH after two years or more if they are on *Culicoides sp.* infested areas and not protected against the flies. Icelandic horses born abroad get IBH at a lower frequency (Bjornsdottir, et al., 2006). In the mid 19th century a number of horses were exported from Iceland to the continent as working horses, but this export diminished in the mid 20th century when machines replaced them. In the late 20th century the export increased again but this time it was mainly riding horses (Björnsson et al., 2004). There are around 230,000 horses of the Icelandic breed in the world today and one third of them are in Iceland, or ~80,000. In the period 1999-2009 exported horses were 1300-2000 each year (Möller et al., 2009) and according to The Icelandic Statistics Bureau (Hagstofan) the profit per year is from 300 millions to one billion (10^9) ISK. The average price for a horse has increased from 300 to 600 thousand ISK from the year 2000 (Möller, et al., 2009). Since horses exported from Iceland are much more likely to develop eczema than Icelandic horses born abroad, IBH is a big financial issue for the horse export. For that reason and also because of animal welfare it is of importance to study the disease and develop a treatment or maybe even a cure.

It has been shown that IBH affected horses have IgE antibodies against salivary gland proteins of midges (Hellberg et al., 2009; Hellberg, et al., 2006; Langner, et al., 2008; A. D. Wilson, et al., 2001) and black flies (Baselgia, et al., 2006; A Schaffartzik, et al., 2009).

The allergens causing IBH are found in the saliva of *Culicoides.* In the salivary glands of the midges there are at least ten allergens or proteins that bind IgE from IBH affected horses. Five of these proteins were suggested to be major allergens because IgE react to them in more than 50% of IBH affected horses (Hellberg, et al., 2006). Isolation of the allergens causing IBH is a prerequisite of developing specific immunotherapy and sensitive diagnostic tests. In this thesis, work with two candidate allergens of IBH is described, Cul nub a21 and Cul nub c4.

In the early days of the IBH project two candidate allergen genes were isolated and their proteins, phosphatase and ribosomal P0 protein expressed and produced in *E. coli* and insect cells, but they seemed to be minor allergens (Althaus et al., 2004; Björnsdóttir, 2008). The lambda cDNA library used for this work was from whole body extract of *C. nubeculosus* and the screening was done with a polyclonal anti horse IgE made in chicken as a monoclonal did not exist. Then a lambda cDNA library from the salivary glands af *S. vittatum* could be obtained (Cupp et al., 1998) and since IgE from 50% of horses with IBH bound proteins from *S. vittatum* (Baselgia, et al., 2006) this was used to make a phage surface display library. Wilson et al 2006 also succeeded in making monoclonal antibodies against the horse IgE for much more accurate screening (A.D. Wilson, et al., 2006). Following this Schaffartzik et al isolated and expressed seven proteins from *Simulium vittatum* in *E. coli.* One of them, Antigen 5 like protein, bound IgE from more than 50% of IBH affected horses (A Schaffartzik, et al., 2009).

Around 1400 salivary glands were picked from cultured midges under a microscope by our collaborators in Berne for obtaining a *C. nubeculosus* lambda cDNA library made by GATC Biotechnology, Germany. From this library Cul n 1 (Ag5) was isolated (Björnsdóttir, 2008) and the

other allergens that have been expressed in insect cells, now five altogether. It was also used to make phage display library that resulted in isolation of ten IgE binding proteins. The allergenicity of these proteins named Cul n 2-11 was verified *in vitro* and *in vivo*. Of these proteins ranging from 12 to 75 kDa *in vitro* analyses showed the prevalence of IgE sensitization of IBH affected horses to the full length of recombinant proteins ranged from 13.0 to 56.5%. Healthy horses did not show relevant IgE binding to the recombinant allergens. Eight allergens were tested *in vivo* (all except Cul n 6 and Cul n 11) and all elicited immediate wheal reactions after 30 minutes in IBH affected horses but no reactions in healthy control horses. The reaction persisted for four hours and in several cases it increased (A. Schaffartzik, et al., 2011).

By using the homologue proteins described in Schaffartzik et al 2011 we isolated the Cul nub a21 and Cul nub c4 from the λcDNA library of *C. nubeculosus* that now are termed cul n 5 (a21) and cul n 10 (c4). Both are unknown salivary glands proteins. When the proteins cul n 5 and cul n 10 were expressed in *E. coli* and tested *in vitro* for allergenicity, a21 (Cul n 5) bound IgE of IBH affected horses in the prevalence 43.5% but c4 (Cul n 10) only 15.2%. Based on this a21 seems to be an important allergen in IBH whereas c4 is maybe less significant, and therefore more emphasis was on work with a21 than c4.

Although the bacterial system is the most straight forward for producing recombinant proteins these proteins are not glycosylated and do not necessarily have the right conformation. For correct conformation or folding and glycosylation they must be produced in insect cells. Soldatova et al 1998 described that when the honey bee allergen hyaluronidase was expressed in *E. coli* the biological activity was only 20-30% of that expressed in insect cell (Soldatova, et al., 1998). Therefore it is necessary to produce allergens correctly glycosylated and test for allergenicity.

Many allergens have successfully been expressed using the baculovirus system in insect cells, such as the hyaluronidase (Api m 2) of honey bee (Soldatova, 1999), a fire ant allergen (Sol I 2) (Schmidt et al., 1996) and four allergens of the mosquito *Aedes aegypti* (Peng et al., 2001; Peng, et al., 2006). Baculoviruses are double stranded, circular, supercoiled DNA molecules in a rod-shaped capsid, and are the most prominent viruses known to affect the insects (Summers et al., 1973). Protein expression systems have been developed mainly based on baculovirus, *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV). Very large genome that is packed into a flexible rodshaped nucleocapsid and can accommodate large fragments of foreign DNA makes AcMNPV suitable for this purpose. The Bac-to-Bac baculovirus system is based on a site-specific transposition of an expression cassette from a donor plasmid into a baculovirus shuttle vector, bacmid, propagated in *E. coli*. recombinant bacmid DNA is isolated from *E. coli* and transfected into insect cells, then viral stocks are harvested from the insect cells and used to infect fresh insect cells for protein expression and analysis (Polayes et al., 1996).

The Bac to Bac system has been used with good results at Keldur but because of temporary difficulties we encountered, resulting in no transfections at all, it was necessary to test everything in the procedure. According to the manufacturer´s protocol (Invitrogen) Cellfectin should be used instead of the Lipofectamine we had used. These two were therefore compared. The results did not show much difference. Cytopathy was detected a little earlier with Cellfectin and expression was detected in

the transfection samples but with Lipofectamine the expression was not detected until first passage. In the end it was probably the batch of DH10Bac competent cells we used that were not working properly.

The pFastBac from Invitrogen contains His tag for protein detection and purification. There had been constant problems in detecting Bac produced allergens in WB with anti His antibodies and the polyclonal rabbit anti His from Santa Cruz that was comparatively best had to be used in high concentration. To facilitate protein detection in the system V5 tag was ligated into the pFastBac HT B. Sequencing showed that the V5 tag was in correct phase with the pFastBac HT B. Along with the V5 tag was another 6xHis tag downstream therefore the pFastBac HT B-V5 contains two 6xHis tags, upstream and downstream. The proteins expressed on the pFastBac HT B-V5 could easily be detected with a monoclonal anti V5. The anti His antibody also bound these proteins much more efficiently than Bac-recombinant proteins without V5 (fig 20). This could be because these proteins have two 6xHis tags instead of one.The Cul proteins are in inclusions and the 6xHis tag upstream might be folded in the proteins. However, when the Cul nub a21 protein with one and two His-tags were purified there was no difference. This might be because when the proteins were purified they were denatured first. It supports that the upstream 6xHis tag might be folded in the protein and therefore can´t bind the anti 6xHis antibodies.

Cul nub a21 gene was ligated into both pFastbac HT B with V5 tag and without. The gene was in correct phase with both vectors and when aligned with published sequences with Blastn (http://blast.ncbi.nlm.nih.gov/) the nucleotide sequence was homologous to a published sequence from *C.sonorensis* AY603639 coding for a domain homologue to penaeidin peptides (Campbell, et al., 2005; Sigurðardóttir, 2008).

rCul nub a21 was expressed successfully in insect cells and two protein bands (~53 and ~43 kDa without the V5 tag, ~55 and ~45 kDa with the V5 tag) were detected in WB with the polyclonal mouse anti a21 made against the recombinant a21 protein produced in *E. coli.* When the 6xHis tag, V5 tag and extra amino acids from the vector are taken into account the predicted molecular mass of the a21 protein is ~50 kDa for a21 without the V5 tag and ~52 kDa for a21 with the V5 tag. Since the anti V5 and the anti a21 antibodies react with the same protein bands and they were never seen in the Sf-9 controls we are quite confident that we have managed to express the a21 in insect cells. The calculated size is somewhat less than what is seen in WB. One possibility is glycosilation but treatment with PNGase did not alter the size. However, it was only tried once and needs further testing.

There are also reports on SDS-PAGE overestimating the molecular weight of proteins and it is known that molecular markers are not all that accurate (Kaufmann et al., 1984). Despite the extra 3-5 kDa discrepancy in size we estimated that the larger band is the a21 protein and the smaller band might be degradation products of the Cul nub a21 proteins.

The Cul nub c4 sequence from phage surface display technology was only 181 base pair and by using blast*n* (NCBI) this part showed 99% identity to the published sequence EU978914 (1339 bp) from *C. nubeculosus* coding for CNSG79 secreted salivary gland protein (Russell, et al., 2009).

Primers were designed after the published sequence and the gene ligated into pFastBac HT B vector and pFastBac HT B vector with V5 tag.

Expression of rCul nub c4 was not detected in WB with the polyclonal mouse anti c4 despite clear cytopathy in Bac-c4 infected Sf-9 cells. Comparison between anti c4 and anti V5 antibodies showed that the anti c4 antibodies did not bind to any specific protein bands and were therefore not used for further analyses. The anti V5 bound to a ~57 kDa protein band but predicted molecular mass of the rCul nub c4 protein is 54 kDa with two 6xHis tags, V5 tag and extra amino acids from the vector. Despite the extra 3 kDa discrepancy in size we assumed it was the correct protein band, because the protein might be glycosilated and also that the SDS-PAGE might be overestimating the molecular weight of the protein. The polyclonal anti c4 antibodies were raised against a small part of the protein, 6 kDa (181 bp), compared to the estimated size of the whole protein is 47 kDa (1254 bp). It reacted well with this small fragment but not to the insect produced whole protein. As these are polyclonal antibodies some of the specific antibodies in the pool should react to the epitopes on the fragment but somehow they do not seem available on the whole length insect produced protein.

The long term aim is to develop an immune therapy against IBH. For the purpose of developing protein vaccines, diagnostic tests or desensitization it is necessary to recognize all the important allergens and be able to produce and purify them. To get the correct picture the proteins should preferably be produced in the natural form or at least with the correct glycosylation for testing the allergenicity and in this case the Baculovirus system is the obvious choice. Furthermore protein purification is often complicated, each single protein has its own character requiring different purification methods. This can be a major obstacle in vaccine production. It is therefore valuable to have the choice of more than one production system for expression and purification.

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6 Appendix

6.1 Primers

Primers for PCR and sequencing

V5 tag:	Nucleotide sequence	Tm°C	GC content %
V5 BamHI fw	5´CTT GGT ACC GAG CTC GGA TCC 3´	63.7	61.9
V5 HindIII rev	5´GCA AGC TTC GTC AGC GGG TTT AAA C 3´	64.6	52.0
Cul nub a21:			
Cnpen BamHI fw	5´ GCGC GGA TCC GCG ATG AAA TTC ATA ATG G 3´	63.3	51.7
Cnpen XhoI rev	5´ GCG CCT CGA GGC TTT TCT TGG ACC TTT TG3´	64.9	55.2
A21-390 fw	5'- GGC TTT ACC GGT GAA TTG -3'	53.7	50.0
A21-671 fw	5'- CAG TGC TGG AAG TTC CTG -3'	56.0	55.6
A21-955 fw	5'- ACC GTC CAA CCG CAT ACA G -3'	58.8	57.9
Cul nub c4:			
CnC4 BamHI fw	5´ GCGC GGA TCC CGC TTCAGACAAAGTGATC 3´	65.9	58.6
CnC4 XhoI rev	5´ GCG CCT CGA GGC TT TCC CTG TAT ATG 3´	62.8	57.7
CNSG79 BamHI fw	5´ GCGC GGA TCC CGC ATGCATCAAAATAAAGG 3´	65.0	53.3
CNSG79 XhoI rev	5'- GCG CCT CGA GGC CCC TGT ATA TGT TGG G -3'	67.4	64.3
CNSG79-210 fw	5'- GAT TAT GCA GCA CCG AGA C -3'	56.7	52.6
CNSG79-561 fw	5'- GAA GGA CTC GGA GTC TAT TCG -3'	59.8	52.4
CNSG79-992 fw	5'- GAA TAA TGG TGG TGT GGC -3'	53.7	50.0

6.2 Nucleotide sequences of *C. nubeculosus*

Cul nub a21 sequence from phage surface display, 1218 bp.

ATGAAATTCATAATGGAATTTTTTTCAATAATTTTCCTTCTTTCCAACTTGTTGGTTTCTGCAAGAGCTT
CTAGTGCCGTTGGTACTAGTCGTGAATTGTATAACCCCGATCCAAAAGCTCACATCAGAGGGGACACTTA
TGAAGAAATCACCCGATTCAATCAAGTTTACTTCACTCAAGAGGCCACTTTTGGAAGTTGTAACAGTAAA
TGTGAAGATTACGCAGAACCAAGAGATAATTTTAAAGAAGACGAGGCTTACAAATTTCCTCAACGTCTAT
GCACCGGCACAATTCACAGTTGCAAAAGTGAATATTCACCCGAATATCATGTGAGACTACCAAAATCTCC
TACGAGTGATGCACGATATTACTATTTTTCAAAACAAATTGGCTTTACCGGTGAATTGTACATTTATGGC
AATAAAAAAGAGCCTGAATACAGATTTGAGATATTCAAAGGTTGGGAGAGGGTTTTTGTAAGATGTGATA
TTTGTCGGTGCTTATGCGATGAGCCGGAAGATAAAAATTCAGTTAGATCATTTTCACTTAACACTGTGAC
AGCAGATAAAGGTGAACATAAAGTTGTAACAGGAATTCAAATTGGAATGAAAGATAAAATATTTTTCTTG
AAAATTCAACAATCACCCTTGTTACCTTTTGGAAGAATTAACAGTGCTGGAAGTTCCTGGAAAGCTGATG
ATCCATCATCAAGTGGAACAAAACTATTCAAATTACAATATAATAAACGAGGTATTCATATGGGAGCAGC
TGAAGCTAATGTAAAATTATCGGAATATGTAGTGACTGCTGTTAAATTCGTTGAACATAAAGGAAACATG
GCACTTGGTGTCGAACTAACACAATTTGACATTTCTACAGGGAAATTACTTGAAAATAGTAAATGGACAT
ACAACTCATTTGCAACCAATATCATTGCCGATGGTAATTCAGGGCACCGTCCAACCGCATACAGTGATCA
TGTTCCTTCCGAAAAAACAAAATTAGGCTTGGTACAATTCAGTCAAAGTCACATGCATGATGACGTTGGT
CAATCAACTGTTCCATTTATCGATTTACAAGCTATTACATCGACTGAATCTGCATTGATTGGTGTAGGTT
TGCTCTTTAGAGGACGTGAATTCAGTGGTGGTTTCATTGCACCAAGTTTAATTACACATCCAATATTTAG
TGGGGAGACAAAAGGTCCAAGAAAA**TAA**

Cul nub c4 sequence from phage surface display, 181 bp.

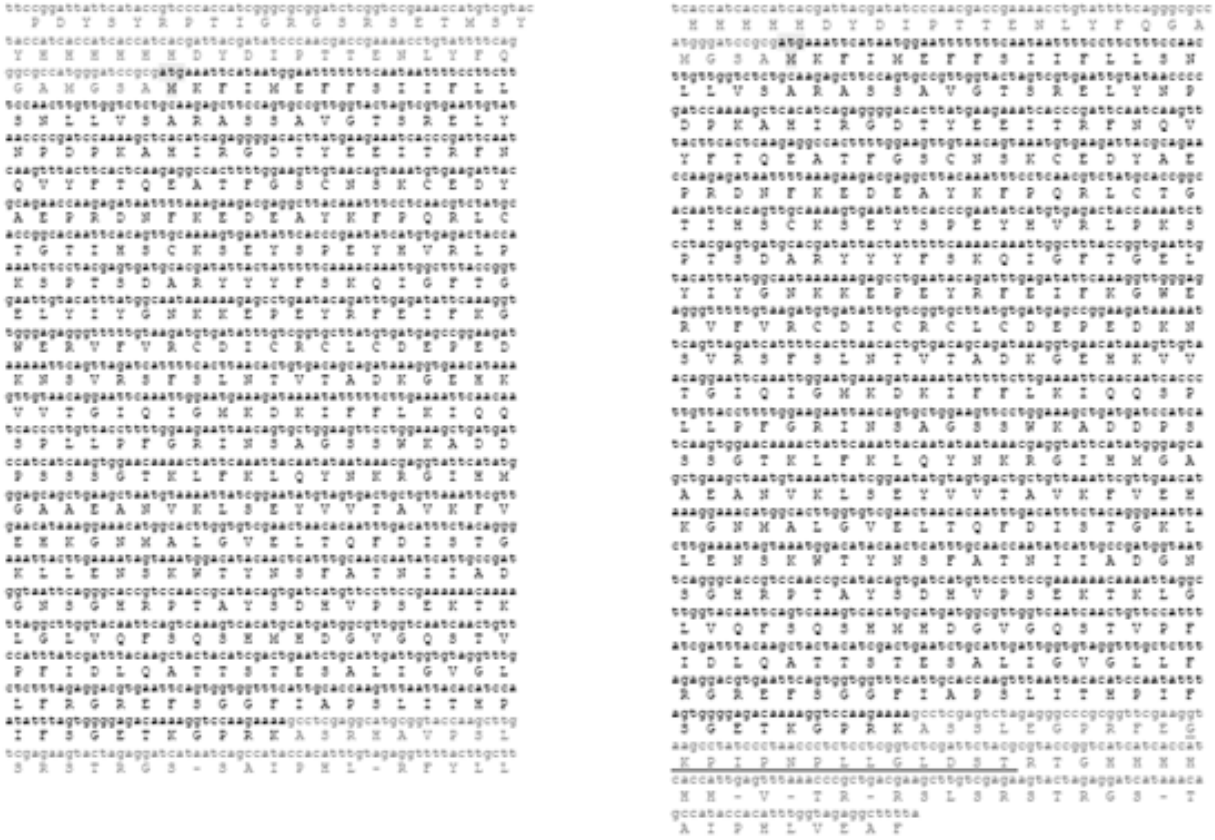
TTCAGACAAAGTGATCTTAAGGATGACGTTGGACAATCAACTGTTCCTTTCATCGATTTGAGATCAATTG
TGTCTACTGGAACGGCATTAATTGGTGTTGGTTTACAGTTCAGGACTCGGGATACTAGTGGTGGGTTTAT
TGCGCCAGTTGTGGTGACTCACCCAACATATACAGGGAAAT

EU978914, *Culicoides nubeculosus* clone CNSG79 secreted salivary protein mRNA, complete cds. 1339 bp.

ATTATTGTCTTTGAAAATTCATTCAGA**ATG**CATCAAAATAAAGGACTCTGGATTCTGATTTCAACCATCA
CATTGATTTCTGGCAATAAACTTCCCTCATTAGGATTCAGTCGTACATTGGACAATGTTGATCCTAAAGA
ACACATAAAAGGAGAAACTTATGAAGAAATCACACGGTTTAATCAAGTTTATTTCACTCAAGAAGCTAAT
TTTGGAAGTTGTAACAGTAAATGTGAAGATTATGCAGCACCGAGACATAATTTTAAAGAAGACAATAGAT
ACAAATTTCCTCAACGACAATGTACTGGAACAATATATAATTGTATAGGTGAAAAAATAACTGAAAAGGA
GCCTGTTATTCGATATCAAATCAAAAAGCCTGAATCTCCAGAGAGTGATAAACGTTATTACTATGTCGAG
CTACCAGTAAATACCGATAAGAAAAAATATCAATATGCCATTATCGAGGGTTCTAATTACACGTATAGGT
ATGTGTATGGAAATAAAGAGCACTCAGATCACACGACAGTCAATTTCGATACTTGGATAAGAGGTTTTGT
TAGATGTGATATCTGTCGTTGTTTGTGTGATGAATCGAAGGACTCGGAGTCTATTCGATCTTTTTCGTTG
AATGCAGTTGAATCAGATACAAAAGATAATAAAGTAGTTATAGGAGTTCAACTTGCACTCAAGGATAAAA
TATTTTATTTTAAAATTCGTCAATCACCTTTGTTACCATTAGGCAAAGCAGATCAAAGAAAACTCTCTTG
GAAGTCTGATGATCCATCATTGAGCTCAGCGAGTAAGGATATTTTCAAATTAGATGTTAATAAGCGTAGT
ATTCATATTGGTTCAGCAACAAGTAATTTATCAGACTTTGTAGTAACTGGTGTTAAATTTGCTGAAATTG
ATGGTAATCTTGCACTTGGAGTTAAACTTACAAAATTTGATGTAATGAGTGGAAAACTTATGAATGATAA
CAATCAATGGATTTATAATTCGTTATCCAAGCAAAAATTGAATAATGGTGGTGTGGCAAATCGTCCAATT
AAATTTACAGATAACCATGCATCGGAAAAACTCGATCTAGGTTTAGTGGAATTCAGACAAAGTGATCTTA
AGGATGACGTTGGACAATCAACTGTTCCTTTCATCGATTTAAGATCAATTGTGTCTACTGGAACTGCATT
AATTGGTGTTGGTTTACAGTTCAGGACTCGGGATACTAGTGGTGGGTTTATTGCGCCAGTTGTGGTGACT
CACCCAACATATACAGGG**TAA**TAAAAAGTATAGTGAATAAAATATTAAATTTATTTGGCTTACTAAAAAA
AAAAAAAAA

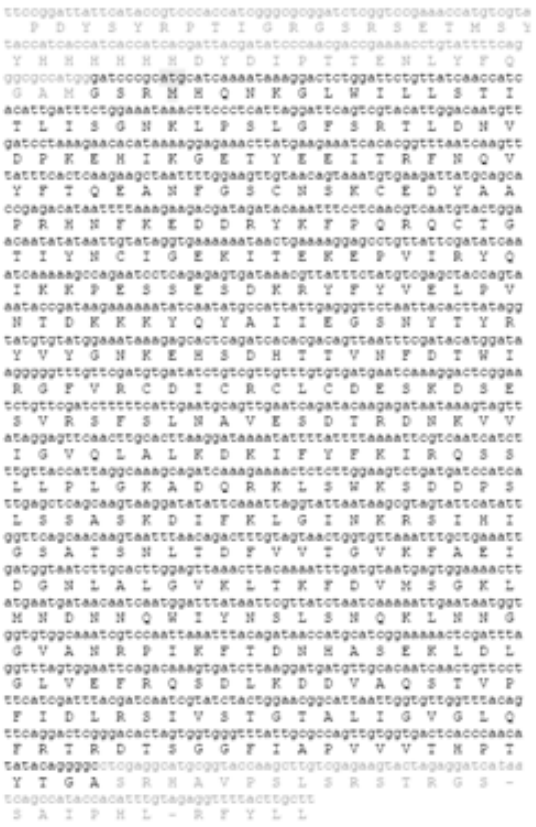
6.3 Sequenced Cul nub a21 and Cul nub C4 and translation into amino acids

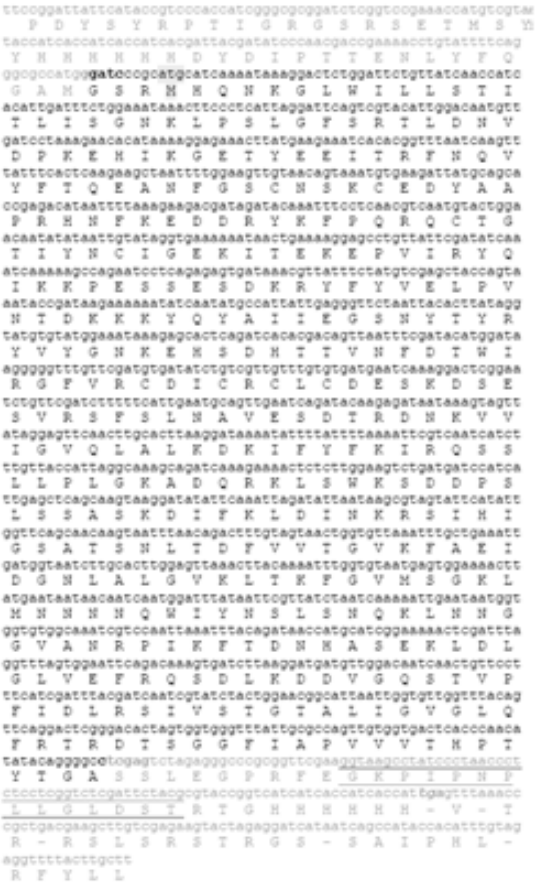
6.3.1 Cul nub a21



pFastbac HT B – cul nub a21 (left) and pFastbac HT B Cul nub a21 – V5 (right), sequenced and translated into amino acid. Yellow ATG is the start codon of the Cul nub a21 gene, the fastbac vector is gray and the V5 Tag is underlined.

6.3.2 Cul nub c4





Fastbac HT B Cul nub c4 (left) and pFastbac HT B cul nub c4 – V5 (right) sequenced and translated into amino acid. Yellow ATG is the start codon of the Cul nub c4 gene, the fastbac vector is gray and the V5 Tag is underlined.