SELECTION, CHARACTERISATION AND ANALYTICAL APPLICATION OF DNA APTAMER AGAINST THE ANAPHYLACTIC TOXIC ALLERGEN, Β-CONGLUTIN, LUP AN 1

Pedro Nadal Polo

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2012

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I CERTIFY that the present study, entitled "SELECTION, CHARACTERISATION AND ANALYTICAL APPLICATION OF DNA APTAMER AGAINST THE ANAPHYLACTIC TOXIC ALLERGEN, β - CONGLUTIN, LUP AN 1", presented by Pedro Nadal Polo for the award of the degree of Doctor, has been carried out under my supervision at the Department of Chemical Engineering of this university, and that it fulfils all the requirements to be eligible for the European Doctorate.

Tarragona, May of 2012

The supervisor of the doctoral thesis

Dr. Ciara K. O'Sullivan

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"*No ha hi res mes perillós que no arriscar-se.*"

Pep Guardiola. 27 May 2009

Seems to be yesterday, when I received a phone call after answering e-mail with a proposal for a position in the University working on SELEX. I could not imagine five years after to became exponentially enriched with master, conferences, publications, and the most important think, to have grown up as a man and as a scientist. Nothing could be possible if my supervisor didn't take risk to enrol me in the aptamer world. My endless gratitude for my advisor; Professor Ciara K. O'Sullivan, who gives me the possibility to develop my career.

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This thesis is dedicated to my parents, without their love, patience, effort, education and, always good guidance I would never achieve anything.

Proud to be your son: "*No podria tenir uns pares millors.*"

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“Pots ser tot allò que tu vulgues,
nomes hi ha un obstacle,
tu mateix”

Als iaios,
el papa, la mama,
lo mano
i a Immi.

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SUMMARY

Lupin has recently been added to the list of allergens requiring mandatory advisory labelling on foodstuffs sold in the European Union, and since December 2008 all products containing even trace amounts of lupin must be labelled correctly. Lupin globulins consist of two major globulins called α-conglutin (11S and "legumin-like") and β-conglutin (7S and "vicilin-like"), and another additional two globulins, γ-conglutin and δ-conglutin, which are present in lower amounts. β -conglutin is the only conglutin currently included in the list of the International Union of Immunological Societies (IUIS), designated as Lup an 1.

The overall objective of these PhD is the selection of aptamers that can detect this allergen. Nucleic acid aptamers are synthetic ligands selected from vast combinatorial libraries through a process referred to as SELEX – Systematic Evolution of Ligand By Exponential Enrichment. Aptamers possess unique chemical and biochemical characteristics, such as: well known chemistry and remarkable stability, moreover, aptamers can be selected against virtually any target and in non-physiological conditions.

In order to achieve the overall objective, a set of subobjectives will be achieved. The first of these involves the elucidation of protocols for the selective extraction of each of the lupin α, β, γ, and δ subunits, resulting in (i) protocols that can be used for selective extraction and isolation of the lupin α, β, γ, and δ proteins from food for subsequent analysis; (ii) standards that can be used in analytical assays and tools; and (iii) target that can be used for the selection of aptamers specific to the β-conglutin subunit.

The core of the work is the selection of aptamers against the allergen Lup an 1 using a SELEX procedure, as well the preparation of protocols that can be used to monitor the evolution of aptamer selection. The functionality of the aptamer is demonstratedby exploiting it in an enzyme linked oligonucleotide assay as well as apta-PCR.

Finally the resulting aptamer candidates that exhibit high affinity are fully characterised, truncated, and the structure of the final truncated aptamer is elucidated.

Specifically outlining the work achieved in this PhD thesis, it is organised into individual chapters.

Chapter 1: Introduction

Statement of the objectives and introduction to the state of the art.

Chapter 2: Extraction, isolation, and characterization of globulin proteins from *Lupinus albus*.

In this chapter we describe a methodology to facilitate the extraction of each of the conglutin subunits using anion-exchange chromatography followed by size-exclusion chromatography. The isolated subunits were characterised using reducing and non-reducing polyacrylamide gel electrophoresis, Western blotting, and peptide mass fingerprinting, all of which revealed that the individual protein subunits are highly pure and can be used as purified targets for SELEX. Furthermore, the extraction protocol can be used for the extraction and selective isolation of each of the subunits from foodstuffs, thus facilitating a highly accurate determination of the β-conglutin concentration.

Chapter 3: DNA Aptamers against the Lup an 1 Food Allergen.

Using in vitro selection, high affinity DNA aptamers to the food allergen Lup an 1, β-conglutin, were selected from a DNA library, 93 bases in length, containing a randomised sequence of 49 bases. Purified β-conglutin from lupin flour was chemically crosslinked to carboxylated magnetic beads and Peptide Mass Fingerprinting confirmed the presence of the β-conglutin. Single stranded DNA was generated from the randomised pool using T7 Gene 6 Exonuclease and subsequently incubated with the functionalised magnetic beads. The captured DNA was then released and amplified prior to a further round of Systematic Evolution of Ligands by Exponential Enrichment (SELEX). The evolution was monitored using an enzyme linked oligonucleotide assay and surface plasmon resonance. Once a plateau in evolution was reached, the isolated DNA sequences were cloned and sequenced. The consensus motif was identified via alignment of the sequences and the affinities of these sequences for immobilised β-conglutin were determined using surface plasmon resonance. The selected aptamer were demonstrated to be highly specific, showing no cross-reactivity with other flour ingredients or with other conglutin fractions of lupin. The secondary structures of the selected aptamers were then predicted using m-fold. Finally, the functionality of these selected aptamers was demonstrated using a competitive assay for the quantitative detection of β-conglutin.

Chapter 4: Analytical application of selected aptamer for β-conglutin.

In this final chapter we report the ultrasensitive detection of the anaphylactic allergen β-conglutin (Lup an 1) using Apta-PCR, exploiting competition between surface immobilized β-conglutin and solution-based target analyte for binding to the S40 β-conglutin aptamer in a competitive assay. Subsequently, by real time PCR analysis the amount of β-conglutin is calculated by amplifying the captured aptamer. The resulting calibration plot has a low detection limit of 6 pM (r^2 0.999) and cross-reactivity against γ-conglutin or gliadin was observed to be negligible.

Chapter 5: Truncation and optimization of selected aptamer for β-conglutin.

Various approaches were used to obtain an optimal truncated aptamer and the essential bases for target binding wereidentified. Through comparing the predicted secondary structures of aptamers using m-fold and GQRS-mapper, a hairpin structure with G-rich loop was determined to be the binding motif and an 11-mer aptamer was evaluated in terms of affinity and specificity using surface plasmon resonance.

Chapter 6: Structure elucidation of truncated 11-mer Lup an 1 aptamer

The structure of the truncated 11-mer Lup an 1 aptamer was further elucidated. Following Reverse Phase HPLC purification, studies were carried out using NMR, HPLC-TOF, comparing the retentions times from the chromatogram with similar structures and with established G-quadruplex containing structures. This study revels that the 11-mer aptamer possessed a more complex structure than a G-quadruplex. Finally, the presence of a dimeric form of the 11-mer aptamer was unequivocally demonstrated using TOF-MS-ES mass spectroscopy and FRET.

Chapter 7: Overall conclusions and future work.

LIST OF PUBLICATIONS

Research articles:

Pedro Nadal, Núria Canela, Ioanis Katakis and Ciara K. O'Sullivan; *Extraction, Isolation, and Characterization of Globulin Proteins from Lupinus albus*; J. Agric. Food Chem., Volume: 59, Issue: 6, Pages: 2752-2758, (2011).

Pedro Nadal, Alessandro Pinto, Marketa Svobodova, Núria Canela and Ciara K. O'Sullivan; *DNA aptamers against the Lup an 1 Food Allergen*; PloS one, Volume: 7, Issue: 4, Pages: e35253, (2012).

Pedro Nadal and Ciara K. O'Sullivan; *Truncation and optimization of selected aptamer for β-conglutin*; Publication in preparation, 2012.

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Book chapters:

Pedro Nadal, Alessandro Pinto, Marketa Svobodova and Ciara K. O'Sullivan; *Aptamers for Analysis: nucleic acids ligands in the post genomic era*; Molecular Analysis and Genome Discovery, Second Edition; John Wiley & Sons, Ltd, 2011.

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LIST OF ABBREVIATIONS

Ab	Antibody
ACE	Angiotensin converting enzyme
ATP	Adenosine-5'-triphosphate
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
SCLC	Small Cell lung cancer
CM5	Carboxymethylated dextran matrix 5
CTLA-4	Cytotoxic T cell antigen-4
CTP	Cytidine triphosphate
ddNTP	Dideoxynucleoside triphosphates
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ddNTP	Dideoxynucleoside triphospate
dsDNA	Double stranded deoxyribonucleic acid
DTT	Dithiothreitol
ED	Eliciting Dose
ELAA	Enzyme-linked aptamer assay
ELISA	Enzyme-linked immunosorbent assay
ELONA	Enzyme-linked oligonucleotide assay
GF	Gel Filtration
GRK2	G-protein-coupled receptor kinase 2
GTP	Guanosine-5'-triphosphate
HCV	Hepatitis C virus

HCV NS5B	Hepatitis C virus nonstructural protein 5B
HPLC	High performance liquid chromatography
HPLC/ESI-MS/MS	High performance liquid chromatography/electrospray ionization tandem mass spectrometry
HPLC-TOF	High performance liquid chromatography time of flight
HRP	Horseradish peroxidase
HTSH	Human thyroid stimulating hormone
IEX	Ion exchange chromatography
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgY	Immunoglobulin Yolk
IUIS	International Union of Immunological Societies
K_D	Dissociation constant
kDa	kiloDalton
KGF	keratinocyte growth factor
LOD	Limit of detection
LOQ	Limit of quantification
MALDI-TOF	Matrix assisted laser desorption/ionization time of flight mass spectrometer
MBs	Magnetic beads
MCP-1	Monocyte-chemotactic protein-1
Mw	Molecular weight
NGF	Nerve growth factor
NTP	Nucleoside triphosphate
PCR	Polymerase Chain Reaction

PDGF	Platelet derived growth factor
PFF	Peptide fragment fingerprint
pI	Isoelectric point
PMF	Peptide mass fingerprinting
PS	Phosphorothioate
PSMA	Prostate specific membrane antigen
PTB	Protein polypyrimidine tract binding protein
RA	Rheumatoid arthritis
RFLP	Restriction fragment length polymorphism
RU	Resonance Units
RNA	Ribonucleic acid
RT-PCR	Real time Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SPR	Surface plasmon resonance
ssDNA	Single stranded deoxyribonucleic acid
TMB	3,3',5,5'-Tetramethylbenzidine
TNFα	Tumor necrosis factor α
VSD	Ventricular septal defect
TTP	Thymidine triphosphate
UTP	Uridine-triphosphate
VEGF	Vascular endothelial growth factor
ε	Extinction coefficient

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CHAPTER 1

INTRODUCTION

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1. Lupin

Lupin is an herbaceous plant of the leguminous family. This plant belongs to the genus *Lupinus*, which includes more than 450 species. Lupin seeds have been used as human food and animal feed since ancient times, having been eaten by the ancient Egyptians, Greeks and by the Mayans in South America, with its high protein content fitting very well in their diet. The only problem was the bitter taste, but years of selective breeding have led to lupin strains with reduced alkaloid content and the "sweet lupins" have been available since the 1920's when the Germans started to cultivate a sweet lupin, which is perfectly suited for human consumption (Ballester, Zacarias et al. 1984).

There are four different species of agricultural interest (Fig. 1.1.), the white lupin (*Lupinus albus*), the blue lupin (*Lupinus angustifolius*), the yellow lupin (*Lupinus luteus*), and the Andean lupin (*Lupinus mutabilis*) (Holden, Faeste et al. 2005). Lupin is considered a source of low-cost protein and can be cultivated in colder climates, making it attractive in comparison to other protein rich plants, like soy (Holden, Faeste et al. 2005) and is attracting attention throughout the world due to high nutritional value (Lilley 1986; Erbas, Certel et al. 2005; Magni, Herndl et al. 2005). The most dedicated country for lupin cultivation is Australia with over 1.000.000 million tonnes/year of the *Lupinus angustifolius*, followed by Germany and France.

Lupinus albus

Lupinus angustifolius

Lupinus luteus

Lupinus mutabilis

Figure 1.1. Lupin species of agricultural interest

1.1. Lupin and its nutritional value

Over the last 10 years, it has been demonstrated that lupin products have an added value over numerous bakery products and have some advantages in comparison to other legumes. White lupin contains high amounts of protein (32.2%), fiber (16.2%), oil (5.95%), and sugar (5.82%). The seed oil is composed of 13.5% saturated, 55.4% monounsaturated, and 31.1% polyunsaturated fatty acids and sucrose constitutes 71% of the total sugar content of seeds. Lupin seeds also contain 3.9 mg/kg of thiamine, 2.3 mg/kg of riboflavin and 39 mg/kg of niacin (Table 1.1.) (Erbas, Certel et al. 2005).

The gross energy, metabolisable energy, and the percent digestibilities of protein and energy for lupin have been found to be 4757 kcal/kg, 2797 kcal/kg, 85.2%, and 66.1%, respectively (Hughes 1988). Lupin or lupin flour is thus used in bread, cookies, pastry, pasta, and sauces, and in beverages as a substitute for milk or soy, and the seeds are used as snacks (Ivanovic, Ballester et al. 1983; Yanez, Ivanovic et al. 1983; Ballester, Zacarias et al. 1984; Depenna, Carreno et al. 1987; Petterson, Sandstrom et al. 1994; Dooper, Holden et al. 2007).

Table 1.1. *Lupinus albus* and *Lupinus luteus* seed composition (C. Martínez-Villaluengaa 2006). Mean values ± standard deviation (n = 4)..

Nutritional components	*L. albus*	*L. luteus*
Protein (g/100 g d.m.)	39.02 ± 0.26	37.93 ± 2.44
Fat (g/100 g d.m.)	14.64 ± 1.15	8.79 ± 0.42
Soluble carbohydrates (g/100 g d.m.)		
Sucrose	4.95 ± 0.12	1.38 ± 0.13
Dietary fibre (g/100 g d.m.)		
Soluble	5.21 ± 0.18	4.90 ± 0.03
Insoluble	34.22 ± 0.08	28.78 ± 0.01
Total fibre	39.42 ± 0.26	33.68 ± 0.04
Starch (g/100 g d.m.)		
Total	3.27 ± 0.23	4.53 ± 0.41
Available	1.78 ± 0.11	1.84 ± 0.13
Vitamins (mg/100 g d.m.)		
α-Tocopherol	0.19 ± 0.01	0.48 ± 0.01
c-Tocopherol	20.10 ± 0.86	11.19 ± 0.63
d-Tocopherol	0.25 ± 0.02	0.38 ± 0.01
Vitamin E activity	2.21 ± 0.11	1.61 ± 0.06
Thiamin	0.36 ± 0.01	1.49 ± 1.12
Riboflavin	0.61 ± 0.04	0.85 ± 0.04
Vitamin C	6.48 ± 0.09	2.56 ± 0.13

UNIVERSITAT ROVIRA I VIRGILI
SELECTION, CHARACTERISATION AND ANALYTICAL APPLICATION OF DNA APTAMER AGAINST
THE ANAPHYLACTIC TOXIC ALLERGEN, B-CONGLUTIN, LUP AN 1
Pedro Nadal Polo
Dipòsit Legal: T. 1067-2012

Lupin seeds and lupin food stuffs are used in many applications as functional and nutracetical ingredients (Duranti, Consonni et al. 2008). An example of the wide potential of lupin, is reported by Grieve *et al.* who describes a banquet given in Hamburg at a botanical gathering, at which a German Professor, Dr. Thoms, demonstrated the multiple uses to which lupin might be put, inviting his colleagues in 1917 to a table covered with a tablecloth of lupin fibre where lupin soup was served; followed by beefsteak, roasted in lupin oil and seasoned with lupin extract, accompanied by bread containing 20 per cent of lupin, lupin margarine and cheese made from lupin albumin, and finally lupin liqueur and lupin coffee. Lupin soap was provided for guests to wash their hands, while lupin-fibre paper and envelopes with lupin adhesive were available for writing (Grieve 1931).

It is difficult to summarize the specific functionalities of lupin proteins, however the most characterised protein isolates also known as Type E and Type F lupin proteins have been shown to possess specific functionalities (Wasche, Muller et al. 2001). Type E, which contains α -, β - and δ -conglutins demonstrate an excellent emulsifying capacity and emulsion stability, as compared to similar fractions from other legume seeds, while Type F has a great solubility over a wide range of pH values and very good foaming activity and foam stability properties (Duranti, Consonni et al. 2008).

On the other hand, lupin protein possesses several biological activities. For example, apart from the observed nutraceutical effects that are attributed to classes of components, such as fibres, starch, oligosaccharides, antioxidants, etc., the blood glucose lowering effect of γ -conglutin has been demonstrated. The capacity of γ -conglutin to interact with the mammalian hormone insulin produces a significant reduction in glycaemic levels in glucose-overloaded rats induced by oral treatment with purified γ-conglutin comparable to the effect obtained with approximately half a dose of metformin, a well known hypoglycaemic drug (Magni, Sessa et al. 2004), thus suggesting a pharmacological response brought about by lupin proteins. In addition to these findings, other biological activities have been attributed to lupin proteins, including plasma cholesterol and triglyceride lowering effects (Sirtori, Lovati et al. 2004), anti-hypertensive properties (Pilvi, Jauhiainen et al. 2006), and inhibition of the angiotensin converting enzyme (ACE) of (Yoshie-Stark, Bez et al. 2004).

1.2. Seed storage proteins of lupin

The seed storage proteins of different species have been studied in detail from the turn of the century, when Osborne (1924) classified them into four groups on the basis of their extraction and solubility characteristics: albumins, soluble in water; globulins insoluble in water but soluble in dilute salt solutions; glutenins, insoluble in the above solutions but soluble in weak acidic or basic solutions; and prolamins, insoluble in the above solutions but soluble in alcohol/water mixtures (Ashton 1976; Shewry, Napier et al. 1995). This classification is still widely used. However, it is well- known that a clear-

cut distinction between these groups of proteins is not always possible, with many proteins showing intermediate solubility behaviours (Franco, Ferreira et al. 1997). The globulin fraction of lupin protein consists of two major globulins called α-conglutin (11S and “leguminlike”) accounting for about 33% of the total protein content in *L. albus* (Duranti, Restani et al. 1981; Foss, Duranti et al. 2006) and β-conglutin (7S and “viciline-like”), which accounts for another 45% (Duranti, Restani et al. 1981; Ferreira, Franco et al. 1999). Finally, there are two additional globulins of minor quantity called γ-conglutin and δ-conglutin which account for 5 and 12% respectively in *L. albus* (Duranti, Restani et al. 1981; Dooper, Holden et al. 2007). Some reports have further subclassified δ-conglutin into δ_1-conglutin, and δ_2-conglutin (Lilley 1986).

The subunit structure of these globulins appears to be complex and differences in the subunits of various Lupinus cultivars and species have been described (Melo, Ferreira et al. 1994). The α-conglutins, also known as legumine like protein 11S, are oligomeric proteins that consist of hexamers of disulphide linked subunits referred to as acidic subunits with high MW, and basic subunits with lower MW. Prior to pro-polypeptide proteolytic conversion to mature subunits, the prevalent form is trimeric. The acidic subunits of α-conglutin, unlike most other 11S globulins, are N-glycosylated (Duranti, Horstmann et al. 1995). The β-conglutin or vicilin like globulin 7S is a trimeric protein in which the monomers consist of a number of polypeptides ranging from 16 to over 70 kDa, with no disulphide bridges linking them. The precursor of the β-conglutin 64 kDa is N-glycosylated. The endogenous cleavage of this family of proteins is well known, such as in pea vicillins, but in lupin seed this phenomenon is greatly enhanced (Duranti, Sessa et al. 1992). The γ-conglutins, a peculiar basic 7S protein, consist of two heterogeneous disulphide linked subunits of 17 and 30 kDa (Scarafoni, Di Cataldo et al. 2001), which are monomerical at acid pH, tending to form tetramers or hexamers at neutral pH. Finally, the monomerical δ-conglutin is similar to other 2S albumins, consisting of two disulphide linked chains of about 4 and 9 kDa (Duranti, Restani et al. 1981). The subunits of all four mentioned classes of proteins arise from the proteolytic cleavage of precursor molecules (Magni, Scarafoni et al. 2007).

1.3. Lupin allergy

Lupin was introduced as a food ingredient in wheat flour in the end of the 1990s in Europe (Moneret-Vautrin, Guerin et al. 1999). Lupin flour is used in biscuits, pasta, sauces and dietetic products sold as milk and soy substitutes (Wittig de Penna 1987; Petterson, Sandstrom et al. 1994; Moneret-Vautrin, Guerin et al. 1999). Due to its emulsifying properties, the use of lupin concentrates in the meat and cold-cut industry has also been reported (Kanny, Guerin et al. 2000).

However, the first documentation of lupin allergenicity came shortly after its' introduction into foodstuffs when Hefle and collaborators reported the case of a 5-year-old girl with peanut sensitivity in 1994 (Hefle, Lemanske et al. 1994). This girl experienced urticaria and angioedema after ingesting pasta fortified with sweet lupin seed flour. Since then several authors have reported several incidences, including a case of acute asthma in a patient with allergy to peanuts (Kanny, Guerin et al. 2000), as well as three subjects who developed work-related allergy symptoms immediately after being exposed to lupin (Crespo, Rodriguez et al. 2001; Campbell, Jackson et al. 2007). Moreover, the majority of the incidences refer to patients with a known allergy to peanut attending specialist medical services, including one patient with peanut allergy who experienced an anaphylactic allergic reaction after eating a hot dog bread (Faeste, Lovik et al. 2004), and another who developed severe respiratory symptoms after eating a pizza (Wuthrich, Mittag et al. 2004). Both the hot dog roll and the pizza had been fortified with lupin flour. Another example details, an 8-year-old asthmatic child, who was also allergic to peanut, who suffered an asthma attack, after manipulating lupin seeds (Moreno-Ancillo, Gil-Adrados et al. 2005).

Due to these and other reports of lupin allergy, in December 2006, lupin and lupin foodstuff products were included in the EU allergen list according to the Commission Directive 2006/142/EC amending Annex IIIA of Directive 2000/13/EC (2006). Since then several population studies have been carried out with the aim of establishing prevalence and to determine the eliciting dose (ED) for lupin allergenicity and its cross-reactivity with peanut, soy, and others legumes (Brennecke, Becker et al. 2007; Peeters, Nordlee et al. 2007; Reis, Fernandes et al. 2007; Wassenberg and Hofer 2007; Holden, Sletten et al. 2008; Lindvik, Holden et al. 2008; Shaw, Roberts et al. 2008; Wuthrich 2008; Dooper, Plassen et al. 2009; Gayraud, Mairesse et al. 2009; Hieta, Hasan et al. 2009; Vinje, Larsen et al. 2009; Fremont, Moneret-Vautrin et al. 2010). One controlled study of peanut allergic patients suggests a clinically relevant cross-reactivity rate of about 30%, but higher (68%) rates have also been reported (Moneret-Vautrin, Guerin et al. 1999). Others have reported that ingested doses of lupin flour known to have triggered clinical reactions, range from 265 to 1000 mg (Kanny, Guerin et al. 2000). More recent studies claim that the eliciting dose for the lupin allergen is about 0.5 mg for allergic adults (Peeters, Koppelman et al. 2009), and this dose can oscillate between 0.2 and 6.4 grams in peanut allergic children (Fiocchi, Sarratud et al. 2009).

Three clinical patterns of allergy to lupin have been described in the literature. The first triggers a reaction via ingestion among individuals allergic to peanuts (Hefle, Lemanske et al. 1994; Moneret-Vautrin, Guerin et al. 1999; Faeste, Lovik et al. 2004). This clinical pattern is typical for peanut allergic patients, which are likely to represent the major risk group. The second is sensitisation via ingestion among individuals with an unknown allergy to peanuts (Matheu, de Barrio et al. 1999; Smith, Gillis et al. 2004). The third is due to sensitisation and triggering via inhalation (Novembre, Moriondo et

al. 1999; Moreno-Ancillo, Gil-Adrados et al. 2005), and occupational exposures among individuals with unknown allergy to peanuts (Crespo, Rodriguez et al. 2001; Parisot, Aparicio et al. 2001; Campbell, Jackson et al. 2007).

Furthermore, there is no definite indication that technological treatments alter the allergenic potential of lupin, although reduction in allergenicity has been reported following autoclaving lupin seeds at 138°C for 30 minutes (Alvarez-Alvarez, Guillamon et al. 2005), and this had been used for the preparation of bread demonstrating a consequent decrease in IgE binding to lupin proteins (Guillamon, Cuadrado et al. 2010).

The reported reactions of lupin allergenicity continue to increase (Moneret-Vautrin 2008; Gayraud, Mairesse et al. 2009; Fremont, Moneret-Vautrin et al. 2010; Moneret-Vautrin, Vitte et al. 2011; Namork, Faeste et al. 2011) and there is not yet any general consensus on the identification of the major allergen/s in lupin (Sirtori, Resta et al. 2011). To date only the **β-conglutin** lupin subunit has been designated as the lupin allergen Lup an 1 by the International Union of Immunological Societies (Goggin, Mir et al. 2008).

1.4. Isolation of lupin Proteins

The first attempt reporting the separation of lupin seed globulins dates back to the pioneering work of Blagrove and Gillespie (1975), where a cellulose acetate electrophoresis technique was used to separate the four main globulin proteins α-, β-, γ- and δ-conglutins found in *L. albus* and *L. angustifolius* (Blagrove RJ 1975) and since then several extraction procedures have been developed. Initially two extraction procedures were tested, the first consisting of alkaline extraction of all proteins and isoelectric precipitation of the α- and β-conglutin globulins and the δ-conglutin albumin. The supernatant of this separation contained γ-conglutin, and could be further purified by selective Zn^{2+} precipitation, leading to more than eight fold enrichment with respect to the amount in the flour. Finally the resuspension of the isoelectric precipitate in saline water/ethanol solution allowed the isolation of δ-conglutin at a very high purity (Sironi, Sessa et al. 2005). Whilst this laboratory procedure results in quite high amounts of proteins and is reported to be easily scalable for industrial applications (Duranti, Consonni et al. 2008) it does not provide targets of maximum purity, as the complete isolation of the target β-conglutin is not achieved.

A rapid method that allows the extraction of the α-, β-, γ- and δ-conglutins in a soluble form in a slightly alkaline buffer has been reported (Melo, Ferreira et al. 1994; Ferreira, Franco et al. 1999).The reported methodology for the isolation of total globulins from legume seeds involves three steps: In the first step seeds are milled and defatted prior to extraction, particularly when seeds of high oil content are being used; in the second step the albumin fraction is removed by treatment of the flour with water and finally the

total globulin fraction is subsequently extracted with a buffered solution containing 10% (w/v) sodium chloride. The extract containing the globulin fraction can be dialysed to remove the sodium chloride added and then purified using Ion exchange chromatography (IEX). IEX is a binding technique that is independent of sample volume provided that the ionic strength of the sample is low and the target molecule is highly charged. Separation in IEX is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium to separate the proteins loaded based on their different charges to give a very high resolution separation with high sample loading capacity. Proteins bind as they are loaded onto a column and then the conditions are altered so that bound substances are eluted differentially as a function of the isoelectric point (pI) for each protein. This elution is usually performed by increasing salt concentration (NaCl) or changing pH, which can be achieved in a stepwise manner, or with a continuous gradient. The net surface charge of proteins when the pH of the solution is below its pI will be positively charged and will bind to a cation exchanger matrix. On the other hand, when the pH is above its pI, it will be negatively charged and will bind to an anion exchanger. Target proteins are concentrated during binding and collected in a purified and concentrated form. There are strong anion exchangers to bind the target if the isoelectric point is below pH 7.0 or unknown and strong cation exchangers that are fully charged over a broad pH range (pH 2-12) as well as weak anion exchangers and cation exchangers that are charged over a narrower pH range (pH 2-9 and pH 6-10, respectively).

1.5. Current methods for detection and quantification of lupin conglutin subunits

There are, several reports of immuno and molecular based detection of lupin, but, to date, none of these selectively detect the Lup an 1 allergen, β-conglutin.

The first report of immune-based detection of lupin was in 1994, describing the preparation of antibodies in rabbits against α-conglutin, β-conglutin and γ-conglutin that had been purified by agarose gel electrophoresis (Bush and Tai 1994).

The next report of the use of antibodies to detect lupin was in 2005, when Holden and collaborators reported the first sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of lupin, where they used a polyclonal rabbit antilupin capture antibody and a biotinylated conjugate of the same antibody for detection. The antibody was highly specific for lupin, apart from minor cross-reactivities to other legumes. The assay had a detection limit of 1 μg/g and was successfully used to quantify total lupin protein in various food matrices (Holden, Faeste et al. 2005). A couple of years later, the same authors published another sandwich ELISA for the detection of lupin proteins in foods. This ELISA consisted of a polyclonal-monoclonal antibody-based sandwich sensitive to both native and processed proteins from *Lupinus angustifolius* and *Lupinus albus*, but did not report any improvement in the detection limit (Holden, Moen et al. 2007). The same year Dooper et al. described the

preparation and characterisation of five monoclonal antibodies (MAbs), one IgG and four IgM antibodies that reacted strongly with protein isolates from both *Lupinus angustifolius* and *Lupinus albus*. All MAbs are directed towards the lupin globulin fraction with Lu11 and Lu18 recognizing α-conglutin, while Lu8, Lu34 and Lu35 recognize β-conglutin. In addition, Lu11 inhibited the binding of IgE from patients with positive skin prick tests to lupin proteins in a competitive ELISA by approximately 30%. Preliminary results showed that Lu11 could be used to develop a sensitive method for the detection of a combination of α-conglutin and β-conglutin in foods with a detection limit of 1 µg/g (Dooper, Holden et al. 2007).

An alternative ELISA reports the preparation of lupin-specific polyclonal anti-serum antibodies raised against lupin flour, that were partially purified from sera by ammonium sulphate precipitation and applied to a sandwich ELISA. Using rabbit antiserum as the capture reagent and sheep antiserum as the detector reagent, with the binding of the antigen-antibody complex being visualized by the addition of commercial rabbit antisheep IgG antibody labelled with alkaline phosphatase, and addition of p-nitrophenyl phosphate substrate, achieving a detection limit of quantification of 1 ppm. Minor cross-reactivity was observed with soy (*Glycine max*) and black bean (*Castanospermum australe*) (Kaw, Hefle et al. 2008). The assay was successfully applied to real sample analysis but is not being specific for β-conglutin.

The latest reports describe the raising of anti-lupin antibodies by immunising a rabbit and a hen with a protein extract from white lupin flour. Hen IgY (immunoglobulin Yolk) was used as coating antibody and rabbit IgG as secondary antibodies in a sandwich format. The ELISA detects proteins from white (Lupinus albus) and blue (Lupinus angustifolius) lupin and, with a lower sensitivity, proteins from yellow (Lupinus luteus) lupin. The ELISA does not show any cross-reactivity with 34 plant species potentially used in lupin containing foodstuffs. Accuracy, repeatability, limit of detection (LOD) and limit of quantification (LOQ) were determined by analysing model biscuits and noodles containing from 0 to 10,000 ppm lupin flour. Lupin flour could be detected in the unprocessed doughs as well as in the processed products down to a spiking level of 1 ppm (Ecker and Cichna-Markl 2012) and is observed to have slight cross-reactivity (≤1.5 %) with peanut. The authors compared sandwich assays using the rabbit IgG as capture and reporter, and the hen IgY as capture and reporter. The IgG ELISA allows the detection of 50 ppm white lupin flour in bread, vegetarian patties and rusk, and with the IgY ELISA, lupin can be detected in vegetarian patties and bread at 50 ppm spiking level and in rusk at a 100 ppm spiking level (Ecker, Ertl et al. 2012).

ELISA kits are commercially available for the detection of lupin. These ELISA kits are provided in a sandwich assay where the microtitre plate is already coated with capture antibody, and examples include the lupin-Check ELISA kit by Imutest, AgraQuant ELISA lupin by Romer Labs, Lupin ELISA kit by Immunolab GmbH and by

ELISA Systems. However, these methods lack in specificity, and do not distinguish between the different globulins.

As an alternative to immuno-based detection, molecular analysis of lupin has also been pursued. In one of the developed assays, genomic DNA sequences coding for conglutin genes were chosen as targets for the detection of lupin, where primer sets and probes were designed for the amplification of 153 bp and 150 bp fragments of α-conglutin and δ-conglutin, respectively, achieving detection levels of 10 mg/kg in baked lupin cookies (Galan, Brohee et al. 2010). A hybridisation probe-based real-time PCR assay, with detection limits as low as 0.1 mg/kg has been reported for the successful detection of the presence or absence of lupin in foodstuff, but lacks in specificity (Demmel, Hupfer et al. 2008). Demmel et al. more recently reported the applicability of this developed real-time PCR method for the sensitive and specific detection of lupin DNA in processed foods. Changes in the amplification efficiency and in detection limit arising from various processing steps such as freezing and baking were observed. For the starting flour, the same limit of detection (0,1 mg/kg) as in raw foods was determined. In baked pizza the detection limit was 1 mg/kg thus demonstrating the suitability of the method for detecting lupin DNA in processed foods (Demmel, Hupfer et al. 2011). Finally, the detection and quantification of lupin flour in wheat flour-based matrices using Real time PCR based on SYBR green has also been reported. Here, the primers used facilitated high PCR efficiencies and did not show any cross-reactivity with DNA extracted from various plant and animal foods. The sensitivity achieved was 7 pg of lupin DNA (Scarafoni, Ronchi et al. 2009).

More recent studies described the use of a silicon-based optical thin-film biochip technology for the simultaneous detection of eight food allergens including celery, almond, oat, sesame, mustard, lupin, walnut and hazelnut on the basis of two tetraplex PCR systems. The detection time was about 30 min after PCR amplification, with the optical thin-film biochip detecting the presence of PCR fragment targets by enzymatically converting the formation of nucleic acid hybrids to molecular thin films. The mass contributed by the thin film alters the interference pattern of light on the biochip surface, resulting in a visible colour change on the chip surface, permitting sensitive, specific and high-throughput detection of allergens in food samples (Wang, Li et al. 2011). The Multiplex ligation-dependent probe amplification (MLPA), able to specifically detect the eight allergens, sesame, soy, hazelnut, peanut, lupin, gluten, mustard, and celery has also been reported. Ligated probes were amplified by polymerase chain reaction (PCR), and amplicons were detected using capillary electrophoresis. Semi-quantitative results were obtained by comparing signals to an internal positive control, and the limit of detection varied from approximately 5 to 400 gene copies, depending on the allergen, with sensitivities similar or better than those obtained with qPCR (Mustorp, Dromtorp et al. 2011).

Some preliminary approaches for the development of a high-performance liquid chromatography/electrospray ionization tandem mass spectrometry method (HPLC/ESI-MS/MS) for the detection and label-free semi-quantification of the main storage proteins of *Lupinus albus* in foods has been reported. This more specific method identifies the main lupin globulins in the tryptic digests of the total protein extracts from white flakes or lupin food products through direct analysis by HPLC/ESI-MS/MS, combined with the use of bioinformatic tools (Locati, Morandi et al. 2006).

In summary, most of these assays are not rapid and the majority of them are only qualitative, being relatively expensive and requiring specialised instrumentation and trained technicians. Moreover none of the reported methods can specifically detect each of, the lupin conglutin subunits, or use specific conglutin standards and thus can completely overestimate the potential toxicity of foodstuffs. As previously mentioned, the rapid detection of allergenic lupin proteins, specifically those subunits that induce a toxic response, is a critical task for producers of raw materials, and food manufacturers due to the risk of inadvertent contamination and consumer safety concerns. The methods developed to date are inherently laboratory based and lack in specificity and there are currently no methods for the specific detection of Lup an 1 allergen, β-conglutin.

2. SELEX and Aptamers for food-safety

In the middle of the 19th century Darwin explained one of the most studied mechanisms of evolution, termed “natural selection” (Darwin 1859), and a century later the first evolutionary experiments with nucleic acids were carried out by Spiegelman using an RNA-dependent RNA replicase for the replication of RNA species (Haruna and Spiegelman 1965; Haruna and Spiegelman 1965; Haruna and Spiegelman 1965; Mills, Peterson et al. 1967). In the beginning of the 1990s, a few years after the development of the polymerase chain reaction (Mullis, Faloona et al. 1986), an artificial *in vitro* selection technique for the isolation of specific nucleic acid sequences called SELEX was individually reported by three laboratories (Ellington and Szostak 1990; Robertson and Joyce 1990; Tuerk and Gold 1990). SELEX (Fig. 1.2.), which is an acronym for Systematic Evolution of Ligands by Exponential Enrichment (Tuerk and Gold 1990) is a procedure in which a very large population of random sequences, in a synthetically produced library of nucleic acids evolves to unique oligonucleotides capable of binding to a desired target. The target-nucleic acid interaction is due to the formation of complex shapes that may act as a scaffold for molecular interactions with the target (Keefe, Pai et al. 2010). These selected nucleic acid sequences, called aptamers derived from the Latin *aptus*, ”to fit” and the Greek word “meros” (part), are artificial ligands (Ellington and Szostak 1990) with the ability to bind to non-nucleic acid target molecules ranging from large complex molecules such as proteins (Tuerk and Gold 1990; Bock, Griffin et al. 1992; Schneider, Feigon et al. 1995; Conrad, Giver et al. 1996), to simple organic small molecules like ATP (Sassanfar and Szostak 1993; Huizenga and Szostak 1995), dyes (Ellington and Szostak 1990; Grate and Wilson 1999), amino acids (Famulok 1994; Geiger, Burgstaller et al. 1996), or even simple small cations (Zhou, Battig et al. 2010), with high affinity and specificity capable of discriminating between enantiomers such as in the case of Thalidomide derivatives (Shoji, Kuwahara et al. 2007).

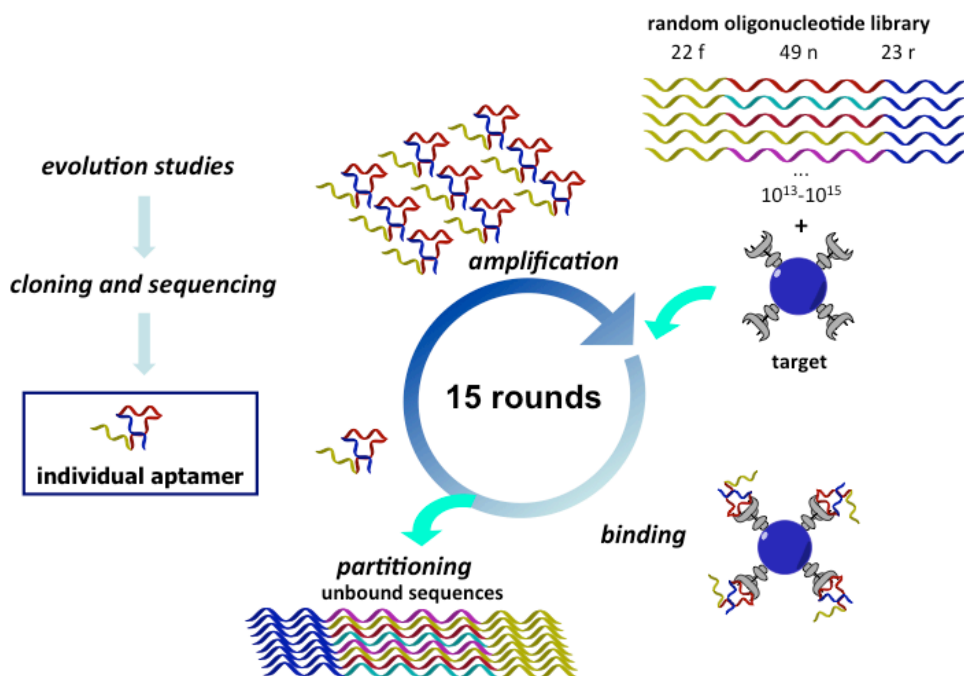

Figure 1.2. SELEX procedure.

Aptamers have many advantageous properties that make them more attractive than other biorecognition elements. Since they are selected by an *in vitro* process the control of the product is very high, meaning that aptamers that bind to a particular region of the target under specific binding conditions, such as salt concentration, pH and temperature can be recovered. Theoretically this *in vitro* selection process facilitates the generation of aptamers against any target. As the SELEX procedure does not require an *in vivo* system, challenges such as toxicity, and lack of immunogenic response observed in animal hosts are overcome (Luzi, Minunni et al. 2003).

Another interesting property of aptamers is their ability to form complex and unique secondary and tertiary structures that confer a high degree of specificity for its target facilitating the possibility to detect small structural changes, such as the presence or absence of a methyl or hydroxyl group (Geiger, Burgstaller et al. 1996; Gopinath 2007).

Once the aptamer is selected, they are easily synthesised and manipulated *in vitro*, and the SELEX process can be automated (Cox, Rajendran et al. 2002). Aptamers also have the ability to renature from a denatured state extending shelf life and, moreover, as aptamer binding can result in a loss of function of the target protein, they are also good candidates for therapeutics (Bunka and Stockley 2006; Lee, Stovall et al. 2006; Keefe, Pai et al. 2010).

Furthermore aptamers can function in a wide range of conditions (buffer or temperature) while the *in vivo* production of antibodies force the antibodies to work only under physiological conditions restricting the range of application and function of antibodies (O'Sullivan 2002; Luzi, Minunni et al. 2003) (Table 1.2.). Finally, aptamers are synthesised chemically and can be provided in more reproducible batches than antibodies giving more consistent results to the final user (Luzi, Minunni et al. 2003).

Table 1.2. Comparison between aptamers and antibodies (O'Sullivan 2002).

Antibodies	**Aptamers**
Limitations against target representing constituents of the body and toxic substances	Toxins as well as molecules that do not elicit good immune response can be used to generate high affinity aptamers
Kinetic parameters of Ab-Ag interactions cannot be changed on demand	Kinetic parameters such as on/off rates can be changed on demand
Antibodies have limited shelf life and are sensitive to temperature and may undergo denaturation	Denatured aptamers can be regenerated within minutes, aptamers are stable to long term storage and can be transported at ambient temperature
Identification of antibodies that recognize targets under conditions other than physiological is not feasible	Selection conditions can be manipulated to obtain aptamers with properties desirable for *in vitro* assay e.g. non-physiological buffer
Antibodies often suffer from batch to batch variation	Aptamers are produced by chemical synthesis resulting in little or no batch to batch variation
Requires the use of animals	Aptamers are identified through an in vitro process not requiring animals
Labeling of antibodies can cause loss in affinity	Reporter molecules can be attached to aptamers at precise locations not involved in binding

To date there are few reports of aptamers selected against food allergens, including a DNA aptamer that recognizes the Asp f 1 allergen of the pathogenic fungus *Aspergillus fumigatus* (Low, Hill et al. 2009), and both RNA and DNA aptamers against the Egg White Lysozyme (Tran, Janssen et al. 2010). Lysozyme was the first example of a food allergen where RNA and DNA aptamers were selected (Cox and Ellington 2001; Huang, Jie et al. 2009), with equilibrium dissociation constants of 2.8 ± 0.3 nM and 0.8 ± 2.0 nM respectively, measured by fluorescence anisotropy at 25ºC (Potty, Kourentzi et al. 2011).

In food safety there have been several reports of aptamers, including the detection of biotoxins such as the mycotoxins fumonisin B (McKeague, Bradley et al. 2010), and endotoxin (Su, Lin et al. 2012), as well as several antibiotics with detection limits ranging from nanomolar to micromolar, e.g. Kanamycin A (Song, Cho et al. 2011) and B (Kwon, Chun et al. 2001), neomycin (Cowan, Ohyama et al. 2000), tetracycline (Berens, Thain et al. 2001), chloramphenicol (Mehta, Van Dorst et al. 2011). Furthermore aptamers have been used for the detection of various bacterial pathogens, including *Salmonella typhimurium* (Singh, Vajpayee et al. 2012), *Escherichia coli* O157:H7 (Lee, Han et al. 2012), *Listeria monocytogenes* (Ohk, Koo et al. 2010) and *Staphylococcus aureus* (Cao, Li et al. 2009).

There is a large diversity of aptamers selected, against a wide variety of targets, where aptamers have been exploited as biomolecular recognition elements in a range of applications, ranging from food quality control to clinical diagnostics (Tombelli, Minunni et al. 2007) (Table 1.3.).

Table 1.3. Targets used for SELEX.

Target	Aptamer	KD	Application	References
Activated protein C	RNA	110 nM	Therapeutics	(Gal, Amontov et al. 1998)
Angiopoietin-2	RNA	3 nM	Therapeutics	(White, Shan et al. 2003)
Aβ4 (Amyloid β4)	RNA	29 nM	Therapeutics	(Rhie, Kirby et al. 2003)
bFGF (Basic fi broblast growth factor)	RNA	350 pM	Therapeutics	(Jellinek, Green et al. 1995)
Cell lung cancer (SCLC)	DNA	NR	Biosensing	(Kunii, Ogura et al. 2011)
Chloramphenicol	DNA	0.8 μM	Biosensing	(Mehta, Van Dorst et al. 2011)
CTLA-4 (cytotoxic T cell antigen-4)	RNA	50 nM	Therapeutics	(Santulli-Marotto, Nair et al. 2003)
Endotoxin	DNA	11.9 nM	Biosensing	(Su, Lin et al. 2012)
ERK2 (Extracellular regulated kinase 2)	RNA	2 nM	Therapeutics	(Seiwert, Nahreini et al. 2000)
Escherichia coli	RNA	NR	Imaging	(Lee, Han et al. 2012)
Ethanolamine	RNA / DNA	6-19 nM	Biosensing	(Mann, Reinemann et al. 2005)
Factor VIIa	RNA	11 nM	Therapeutics	(Rusconi, Yeh et

				al. 2000)
Factor IXa	RNA	600 pM	Therapeutics	(Rusconi, Scardino et al. 2002; Rusconi, Roberts et al. 2004; Que-Gewirth and Sullenger 2007)
Fumonisin B-1	DNA	100 nM	Biosensing	(McKeague, Bradley et al. 2010)
GRK2 (G-protein-coupled receptor kinase 2)	RNA	101 nM	Therapeutics	(Mayer, Wulffen et al. 2008)
Haemaglutinin from *human influenza virus*	RNA	0.18 nM	Therapeutics	(Gopinath, Misono et al. 2006)
HCV NS5B	RNA	1-2 nM	Therapeutics	(Biroccio, Hamm et al. 2002; Bellecave, Andreola et al. 2003)
HNE (Human Neutrophil Elastase)	RNA	10-71 nM	Therapeutics	(Smith, Kirschenheuter et al. 1995)
HNPS (Human nonpancreatic secretory phospholipase A2)	RNA	2 nM	Therapeutics	(Bridonneau, Chang et al. 1998)
HTSH (human tyroid stimulating hormone)	RNA	2-3 nM	Biosensing	(Lin, Nieuwlandt et al. 1996)
Human complement C5	RNA	2-40 nM	Therapeutics	(Biesecker, Dihel et al. 1999)
IgE	RNA	9 nM	Therapeutics	(Wiegand, Williams et al. 1996)
IgE	DNA	134 nM	Biosensing	(Wang, Lv et al. 2008)
Kanamycin	DNA	78.8 nM	Biosensing	(Song, Cho et al. 2012)
KGF (keratinocyte growth factor)	RNA	3-400 pM	Therapeutics	(Pagratis, Bell et al. 1997)
L-Selectin/IgG (LS-Rg)	RNA	450 pM	Therapeutic and diagnostic	(Watson, Chang et al. 2000)
L-Selectin/IgG (LS-Rg)	DNA	2 nM	Therapeutic	(Hicke, Watson et al. 1996)
Listeria monocytogenes	RNA	NR	Biosensing	(Ohk, Koo et al. 2010)
Lysozyme	DNA/RNA	0.8nM/ 2.8nM	Biosensing	(Potty, Kourentzi et al. 2011)
mAb 20 (anti-insulin receptor antibody)	RNA	30 nM	Therapeutic	(Lee and Sullenger 1996)
MCP-1 (Monocyte-chemotactic protein-1)	RNA	180 pM	Therapeutic	(Rhodes, Smithers et al. 2001)
NF-kB p50 protein	DNA	NR	Biosensing and imaging	(Yang, Li et al. 2003)
NGF (Nerve growth factor)	RNA	200 nM	Therapeutic	(Binkley, Allen et al. 1995)
NPY (Neuropeptide Y)	RNA	370 nM	Therapeutic	(Proske, Hofliger et al. 2002)
Ochratoxin A	DNA	NR	Biosensing	(Chen, Fang et al. 2012)
Oncostatin M	RNA	7 nM	Biosensing	(Rhodes, Deakin

				et al. 2000)
PDGF (Platelet derived growth factor)	DNA	100 pM	Therapeutic	(Green, Jellinek et al. 1996; Akiyama, Kachi et al. 2006)
P-Selectin	RNA	16-710 pM	Therapeutic	(Jenison, Jennings et al. 1998)
PSMA (prostate-specific membrane antigen) (sPSM)	RNA	2 nM	Therapeutic and imaging	(Lupold, Hicke et al. 2002)
PTB protein (Polypyrimidine Tract Binding)	RNA	NR	Therapeutic	(Anwar, Ali et al. 2000)
Raf-1	RNA	150 nM	NR	(Kimoto, Shirouzu et al. 2002)
SalmonellaTyphimurium	DNA	NR	Biosensing	(Singh, Vajpayee et al. 2012)
Staphylococcus aureus	DNA	NR	Biosensing	(Cao, Li et al. 2009)
Streptavidin	DNA	85 nM	Biosensing	(Stoltenburg, Reinemann et al. 2005)
Tenascin-C	RNA	5 nM	Therapeutic	(Hicke, Marion et al. 2001)
Thrombin	RNA / DNA	3 nM / 83 pM	Therapeutic and biosensing	(Bock, Griffin et al. 1992; Tasset, Kubik et al. 1997; White, Rusconi et al. 2001)
Thrombin	RNA	37 nM	Therapeutic and biosensing	(Kubik, Stephens et al. 1994)
Transcription factor E2F	RNA	NR	Therapeutic	(Martell, Nevins et al. 2002; Nimjee, Rusconi et al. 2005)
***Trypanosoma brucei* (flagellar pocket protein)**	RNA	60 nM	Therapeutic	(Homann and Goringer 1999)
***Trypanosoma brucei* VSD (Ventricular Septal Defect)**	RNA	160 pM	Biosensing	(Lorger, Engstler et al. 2003)
***Trypanosoma cruzi* cell adhesion receptor**	RNA	100 nM	Biosensing	(Ulrich, Magdesian et al. 2002)
VEGF (Vascular endothelial growth factor)	RNA	130 pM	Therapeutic and biosensing	(Ruckman, Green et al. 1998)
Zn^{2+}	RNA	1.2 nM	Biosensing	(Ciesiolka, Gorski et al. 1995)

NR means not reported

2.1. Target preparation

Once the target for SELEX is obtained it is necessary to characterise it before starting the SELEX procedure in order to develop highly specific aptamers against the desired target protein. Thus, following purification, isolated protein targets are generally characterised including, for example, reducing and non-reducing polyacrylamide gel electrophoresis, Western Blotting, and Peptide Mass Fingerprinting to ensure that the target is present in a highly purified form.

In Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), proteins are primarily denatured using both reducing and non-reducing conditions. Proteins become unfolded and coated with SDS, a detergent that infers the protein molecules with an overall negative charge that is proportional to the length of the polypeptide chain. The proteins are loaded onto a gel matrix and placed in an electric field, with the negatively charged molecules migrating towards the positively charged electrode being separated according to their molecular mass. The size of the protein can be estimated by comparison of its migration distance with that of a standard of known molecular weight, confirming its' purity.

Peptide Mass Fingerprinting can be used for further characterisation of the protein target. The isolated proteins are digested with a protease, an enzyme capable of digesting proteins at specific places due to their amino acid sequence; an example being trypsin, which excises at arginine or lysine without a proline behind the c-term, thus cutting the protein chain into small peptides. The resulting peptide mixture, extracted from the polyacrylamide gel is then spotted on a MALDI plate for a MALDI-TOF equipment (Matrix-assisted laser desorption/ionisation-Time-of-Flight mass spectrometer) and fired with a laser beam producing sample ionization and the peptides obtained fly to the detector due to the electric current applied and finally the peptide mixture is analysed by mass spectrometry, generating a peptide mass profile characteristic for the excised protein.

This experimental profile is then compared to the theoretical masses derived from the digestion at the same enzyme excising sites of all protein sequences contained on the Swiss-Prot or NCBI database (Rappsilber, Moniatte et al. 2003). The proteins in the database are then ranked according to the number of peptide masses matching their sequence within a given error tolerance in mass. This process is called peptide mass fingerprinting (PMF) (Rappsilber, Moniatte et al. 2003). A protein is generally considered identified with sufficient confidence when at least five peptide masses are matched with a mass accuracy better than 30 ppm, 15% of the sequence is covered and the next best database hit shows significantly less agreement with the experimental data (Rappsilber, Moniatte et al. 2003).

2.2. Target immobilisation

The method of target immobilisation to a solid support will depend on the partitioning methodology used during SELEX. During partitioning the specific sequences are separated from the non-specific equences. The selected method should maintain the target aptatopes exposed to the aptamers (Balamurugan, Obubuafo et al. 2008). The most widely used platform is magnetic particles with different surface chemistries including amine, thiol, epoxy, streptavidin or biotin (Hianik, Ostatna et al. 2007; Wochner, Menger et al. 2008; Wang, Yang et al. 2009). Alternatively SELEX can be performed against a non-immobilised target, using for example, nitrocellulose filters for partitioning and having the advantage of having the target in its' natural state with a higher number of potential aptatopes.

There are several techniques that allow confirmation of target immobilisation. For example PMF can be used to qualitatively determine if the protein of interest has been immobilised on the magnetic bead surface. Immobilised protein is digested with a protease to generate a peptide mass profile characteristic for the excised protein that is then compared with all the proteins contained in the Swiss-Prot or NCBI data-bases. Furthermore, using ELISA the amount of target attached to the surface can be determined. Other methods that can be used include zetapotential, IR or NMR but the applicability will always depend on the size of the molecule and the surface chemistry of the support where the target is attached.

2.3. SELEX Library

The design of the SELEX library depends on the SELEX procedure. Typically an aptamer library consists of 10^{13}–10^{15} random oligonucleotides consisting of a diverse sequence of random nucleotides flanked by constant regions of nucleotides that will be used to bind the reverse and forward primer respectively for amplification using PCR after each selection round.

The complexity of the library generated can be calculated relatively easily, assuming that the manufacture of the aptamer has been random, by COMPLEXITY=y^N, where N is the number of oligonucleotides in length, generated from y different nucleotides which in case of DNA are 4; adenine, guanine, cytosine and thymine. Therefore, if N=35 and y=4 the maximum diversity, referred to as the "sequence space", is around 10^{21}. However, for practical purposes the maximum number of sequences that can be screened in one library is 10^{13}–10^{15} (Sampson 2003). Moreover the amplification step by the DNA polymerase is not 100% accurate and will introduce further mutations into selected oligonucleotides offering the possibility to increase diversity during selection.

For the selection of RNA aptamers, the random DNA oligonucleotide library has to be transcribed into RNA before starting the first round of a RNA SELEX. For this reason a sense primer with an extension at the 5'-extreme containing the T7 promoter sequence

and an antisense primer are necessary to amplify the ssDNA library by PCR. The final dsDNA is then *in vitro* transcribed using the T7 RNA polymerase.

2.4 Selection Procedure

The *in vitro* selection procedure involves three principal consecutive steps: binding, partitioning and amplification. The binding step consists of the interaction between the candidate library and the target molecule. In the partitioning step the mixture of candidates with high affinity are separated from the others with low or no affinity against the target molecule, and finally the amplification of the bound sequences.

2.4.1. Binding

The first step during a SELEX round is the incubation of the target with the oligonucleotide pool. This incubation is carried out under specific temperature and buffer conditions. The main goal in this step is to obtain library members with a high-affinity to the target molecule. Moreover, it is possible to improve the possibilities of selecting high affinity aptamers, by increasing the stringency via reduction of the target concentration or changing the binding and washing conditions (Marshall and Ellington 2000). Additionally, to reduce the chances of evolving non-specific binders, those sequences of the library that bind to the support material (e.g. affinity chromatography column, magnetic beads, nitrocellulose filters etc.) can be excluded, performing a pre-incubation without the target before each round in a process termed as negative selection (Ellington and Szostak 1992; Ellington and Szostak 1992). In a similar manner, to reduce the cross-reactivity of the selected aptamer, a pre-incubation step in the presence of potentially interfering molecules that are structurally similar to the target, can be carried out in an additional step referred to as counter selection (Jenison, Gill et al. 1994; Fitzwater and Polisky 1996).

2.4.2. Partitioning methodology

During the partitioning step the bound oligonucleotides are separated from molecules not bound to the target. Several partitioning methods have been described including affinity chromatography with immobilisation of the target on a sepharose or agarose column (Nieuwlandt, Wecker et al. 1995; Liu and Stormo 2005; Tombelli, Minunni et al. 2005), affinity tags (Dobbelstein and Shenk 1995), centrifugation (Rhie, Kirby et al. 2003), surface plasmon resonance (Misono and Kumar 2005), capillary electrophoresis (Mosing, Mendonsa et al. 2005), atomic force microscopy (Miyachi, Shimizu et al. 2010) or flow cytometry (Yang, Li et al. 2003). The use of magnetic beads with magnetic separation is also widely used as a useful tool for the separation of target and nucleic acids (Bruno and Kiel 2002; Lupold, Hicke et al. 2002; Murphy, Fuller et al. 2003), as this method only requires small amounts of target and is simple to handle.

Whilst the immobilisation of the target on a specific matrix allows an effective separation, protein immobilisation can destroy key features of some aptatopes as well as reducing the mobility of the protein. The use of ultrafiltration without target immobilisation is thus an alternative method used for partitioning (Tuerk and Gold 1990; Schneider, Gold et al. 1993; Fitzwater and Polisky 1996; Bianchini, Radrizzani et al. 2001), and although this nitrocellulose filter technique is very widely reported, there are significant non-specific interactions with the nitrocellulose membrane combined with large losses of target-bound oligonucleotides.

An optional partitioning strategy is based on detecting fluorescence via flow cytometry (Davis, Abrams et al. 1997; Blank, Weinschenk et al. 2001; Yang, Li et al. 2003), or alternatively, using surface plasmon resonance (SPR), which provides binding efficiency information and on-line evaluation during the selection process (Misono and Kumar 2005). Atomic force microscopy (AFM) can dynamically detect the adhesion or affinity force between a sample surface and a cantilever, a feature that is useful for the selection of high affinity aptamers (Yusuke, Nobuaki et al. 2009). Electrophoretic separation (Jensen, Atkinson et al. 1995; Yao, Adelman et al. 1997; Goodman, Velten et al. 1999; Golden, Collins et al. 2000) or centrifugation (Homann and Goringer 1999; Rhie, Kirby et al. 2003) are further examples of tools for partitioning.

More recent studies report on the use of sophisticated microfluidics systems that enhance the efficiency of selection (Ahn, Jo et al. 2011) with immobilisation-free screening of aptamers assisted by graphene oxide (Park, Tatavarty et al. 2012).

2.4.3. Amplification and ssDNA Generation

The amplification of the selected sequences is carried out using PCR. There are several techniques for the preparation of ssDNA after PCR amplification of a DNA aptamer. One example employs assymmetric PCR amplification which produces ssDNA due to the unequal concentration of sense and antisense primers used in the reaction mixture. However, since it generates both ssDNA and dsDNA, subsequent purification of ssDNA is required (Boiziau, Dausse et al. 1999). The need for the subsequent purification of ssDNA can be circumvented by other alternative methods of generating ssDNA, for example using biotin–streptavidin magnetic beads separation (Stevenson, Baxter et al. 2008), where during PCR one of the primers used is biotinylated and the resulting biotinylated PCR product is immobilised on streptavidin coated magnetic beads. Due to the high affinity of the streptavidin towards the biotin, the desired non-biotinylated strand is separated from the biotinylated strand by alkaline (NaOH) or temperature treatment.

Another option for the preparation of single stranded DNA is the use of enzymes such as T7 Gene 6 exonuclease (Ruan and Fuller 1991; Nikiforov, Rendle et al. 1994) and Lambda exonuclease via modified primers (Higuchi and Ochman 1989; Kujau and

Wölfl 1997; Jones, Clancy et al. 2006; Avci-Adali, Paul et al. 2009). T7 Gene 6 exonuclease hydrolyzes one strand of DNA non-processively in the 5´ to 3´ direction. The phosphorothioate (PS) bond substitutes a sulphur atom for non-bridging oxygen in the phosphate backbone of an oligo. This modification renders the internucleotide linkage resistant to nuclease degradation. Phosphorothioates can be introduced at either the 5'- or 3'-end of the oligo to inhibit exonuclease degradation. The forward primer is phosphorothioated in order to prepare single stranded DNA following PCR. Alternatively Lambda exonuclease is an enzyme involved in the repair of double-stranded breaks of the viral DNA (Dapprich 1999), which originates from bacteriophage l and Lambda exonuclease selectively digests the phosphorylated strand(s) of dsDNA from the 5' to the 3' end. A comparison of these different techniques has been evaluated and compared in terms of cost, required time and efficiency (Svobodova, Pinto et al. 2012).

2.5. SELEX Evolution

Monitoring the evolution of the pool affinity during the selection process allows control and adjustment of the selection pressure and stringency to achieve the desired properties of the selected aptamers. There are several methods that have been reported for monitoring of evolution:

Radiobinding Assays. Probably the most commonly used technique for monitoring evolution uses radioactively labelled nucleic acids (Jeong, Eom et al. 2001; Sayer, Ibrahim et al. 2002). In a technique known as restriction fragment length polymorphism analysis (RFLP), the corresponding population of the candidates from each round are exposed to an enzymatic cocktail of endonucleases, all of which recognise a particular short restriction site. The results are analysed by electrophoresis on a denaturing polyacrylamide gel after the enzymatic digestion of the double-stranded labelled DNAs. During the final rounds, if selection is achieved, one single RFLP profile should be obtained indicating that the oligonucleotide population diversity converges to unique single molecule domains (Pestourie, Cerchia et al. 2006).

Surface Plasmon Resonance. The confirmation of evolution during SELEX can also be achieved using Surface Plasmon Resonance a technique that is extremely useful for analysing various biochemical interactions, including RNA–protein interactions, and allows real-time monitoring of complex formation (Park, Myszka et al. 2000; Katsamba, Myszka et al. 2001). SPR can also be used for monitoring and enrichment of binding species from a library by using a small amount of immobilised target that allows the identification of binding nucleic acid sequences (Khati, Schuman et al. 2003; Pileur, Andreola et al. 2003). Moreover, the use of SPR to directly monitor SELEX has been reported (Misono and Kumar 2005), and it is also possible to check the specificity of the selected DNA pool by immobilising different targets as negative controls.

ELONA. While to date, this type of assay has only been reported for detection, it can also be used for monitoring of evolution. The first ELAA (enzyme-linked aptamer assay) or ELONA (Enzyme Linked Oligonucleotide Assays) assay were reported by Drolet *et al.* (Drolet, MoonMcDermott et al. 1996) for the detection of human vascular endothelial growth factor on a microtiter plate. Since then many similar assays have been reported for describing the use of aptamers in similar assays by using either labelled or immobilised nucleic acid molecules as capturing or detecting agents, similar to antibodies used in Enzyme-Linked ImmunoSorbent Assays (ELISAs) (Preiser, Elzinger et al. 2000; Yamamoto and Kumar 2000; Ramos, Pineiro et al. 2007). These types of assays can be used to detect the presence of an analyte, where the aptamer is the capture molecule or to detect the presence of aptamers by using analytes as a capture molecule which would be the case when used for monitoring evolution.

Fluorescence. In the same manner as in radio labelling, fluorescent dyes can be introduced by PCR (Stoltenburg, Reinemann et al. 2005), or, alternatively using OliGreen, which is specific for single-stranded DNA and allows the quantification of small amounts of DNA in the pg/mL range using a standard spectrofluorimeter (Wochner and Glokler 2007).

Pilot PCR. Following the partitioning of bound from unbound DNA, the selected oligonucleotide pool is amplified using pilot PCR. In pilot PCR a small aliquot of the SELEX pool is amplified in the range of 5 to 20 PCR cycles in order to decide the desired conditions of amplification to maintain the same amount of DNA for each cycle of SELEX. This procedure allows the maintenance of the same number of molecules for all SELEX rounds and assuming that the diversity of a nucleic acid library is dramatically reduced during the selection process, the pilot PCR. Can be used to show the increased amount of specific target binders versus the decreased amount of non-specific binders. Pilot PCR is useful for the monitoring of SELEX during selection by treating the negative selected pool in the same manner via amplification of the unspecific sequences that bind to the negative matrix (without protein attached), also facilitating the monitoring of the decreasing amount of unspecific sequences and the increasing amount of specific sequences, as more PCR cycles are required for the amplification of the negative selected pool and less for the positive selected pool. This approach allows facile identification of the plateau in affinity achieved during the SELEX procedure by the aptamer candidates.

2.6. Cloning and sequencing

Once evolution has been established, cloning and sequencing can be carried out to define the consensus motif. After several rounds of SELEX (8 to 15), the pool obtained in the last SELEX round is cloned into a bacterial vector and individual colonies are sequenced (Stoltenburg, Reinemann et al. 2005). The number of library members after a complete SELEX method can be expected to be from 1 to 1.000.000 (Conrad, Baskerville et al. 1995), with the variation in this number depending on parameters including the nature of the target and target concentration. The main goal of the cloning procedure is to split the pool of library members into single members cloning into a bacterial vector to obtain individual colonies with single members (Stoltenburg, Reinemann et al. 2007). The bacterial plasmid is then extracted for sequencing.

2.6.1. Cloning

The procedure of cloning starts with the amplification of the desired SELEX round. The *Tfi*-polymerase has a no template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The 3'-adenylation of the PCR product is achieved by using an excess of the *Tfi*-polymerase in a 10:1 ratio, or by including a 30 minute extension at 72°C after the last PCR-cycle. The linearised vector used, pCR®2.1-TOPO® will have a single, overhanging 3´ deoxythymidine (T) residue which allows the PCR inserts to ligate efficiently with the plasmid vector. Finally, the Topoisomerase I that binds to duplex DNA at specific sites cleaves the phosphodiester backbone after 5'-CCCTT in one strand (Shuman 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3'-phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. To complete ligation of the insert with the vector the phospho-tyrosyl bond between the DNA and enzyme is subsequently attacked by the 5'-hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman 1994).

Following the construction of the recombinant vector, it is inserted into competent *E.coli*. This procedure is achieved by electroporation or by chemical transformation of the cells. The constructed vector contains the genes to produce cells resistant to ampicillin and kanamycin and for expression of β-galactosidase, an enzyme that results in a product of blue colour after the addition of X-gal to the culture medium. These genes allow the discrimination between the transformed cells that have incorporated the plasmid containing the inserted aptamer candidate and non-transformed cells.The cells with the plasmid incorporated are viable in ampicillin and kanamycin medium, whilst the aptamer candidate inserted into the coding sequence of the gene results in non-expression of the enzyme, facilitating differentiation between the cells not containing (blue cells) and those containing the candidate aptamer sequence (white or light blue).

2.6.2. Sequencing

The next step is the determination of the nucleotide sequences in ssDNA. The most commonly used technique is Sanger's method (chain-terminator method) (Sanger and Coulson 1975), which is also known as dideoxy sequencing. The key to Sanger's sequencing method is the chemistry of dideoxynucleotides (ddNTP). This method uses dideoxynucleotides (ddNTPs) in addition to the normal nucleotides (dNTPs) found in DNA. The difference between ddNTPs and dNTPs is, the hydrogen group on the 3' carbon instead of a hydroxyl group (OH). These modified nucleotides stop the addition of further nucleotides during PCR. In manual Sanger sequencing four different PCRs reactions are run in parallel using one nucleotide terminator in each PCR. The four PCR products are then analysed by gel electrophoresis, separated according to the fragment lengths and the sequence identified. However, this is a very laborious and error-prone technique and the development of labelling techniques allowed single base labelling using fluorochromes, facilitating automatation of the method. The technique was vastly simplified by reducing to a single PCR and detecting each of oligonucleotide labelled with four different fluorophores. Currently, there is a lot of focus on what are referred to as next generation sequencing, with commercial examples including the Roche 454, Illumina Genome Analyzer (GA), and Applied Biosystems (ABI) SOLiD. These next Generation Sequencing techniques parallelise the sequencing process, producing thousands or millions of sequences at once (Cho, Xiao et al. 2010; Nitsche, Kurth et al. 2007), and aims at sequencing the genome for a target price of less than one thousand dollars.

2.6.3. Analysis of clone libraries

A consensus motif can be defined as an average nucleotide sequence; each nucleotide is the most frequent at its position in the sequence. Analysis of the conserved regions is used for the prediction of the secondary structure of aptamer candidates using specialised programs developed for this purpose (Davis, Janji et al. 1996). For the analysis of the sequences obtained bioinformatic tools are used to find the consensus sequence. There are many programs available, and the most widely used program is CLUSTAL. The first CLUSTAL program written by Des Higgins more than 20 years ago, was designed to perform efficient alignment on PCs (Higgins and Sharp 1988; Higgins and Sharp 1989). Since then the program has been improved and is now a powerful tool that is widely used for multiple alignment and for the preparation of phylogenetic trees, and harnesses a memory-efficient, recursive alignment algorithm (Myers and Miller 1988) with the progressive alignment strategy introduced by Feng and Doolittle (Feng and Doolittle 1987) and Willie Taylor (Taylor 1988). The essence of progressive alignment is to align the most-closely related sequences first and the difficult divergent ones last. The pre-comparison uses a rapid FASTA-like word search, and the dendrogram was constructed using the UPGMA method (Unweighted Pair Group Method with Arithmetic Mean) (Wilbur and Lipman 1983). In 1992 CLUSTAL

incorporated profile alignments and the facility to generate trees from the alignment using the fast Neighbour-Joining method (Higgins, Bleasby et al. 1992).

In summary, the CLUSTAL software is a powerful engine capable of aligning the data obtained in the sequencing step. It is based on the concordance between different sequences and some specific motif sequences that differ by the position of single nucleotides. Using this type of analysis, the consensus motif of the sequences can be identified and at the same time, groups based on homology can be observed on the phylogenetic tree.

3. Aptamer characterisation

Following the confirmation of binding sequences aptamers can then be truncated eliminating the parts of the sequence identified not to be taking part in target recognition. Shortened aptamer sequences are determined and synthesised on the basis of the predicted secondary structure, with the objective that truncation of the aptamer will result in a lower cost aptamer and can also increase the specificity or the affinity of the aptamer.

3.1. Truncation studies

A full-length aptamer usually has three functional regions. The region that plays the role of contacting the target is normally approximately 10–15 nucleotides long, and has secondary structures such as hairpin loops, G-quartet loops, bulges, or pseudoknots. Another region contains nucleotides that do not directly contact the target but play an important role in supporting the interactions between the contacting nucleotides and the target. The third region comprises the nucleotides that do not bind to the target nor support the binding of the contacting nucleotides to the target (Gold, Polisky et al. 1995). They are regarded as nonessential nucleotides. It is always desirable to truncate the aptamer to eliminate the nonessential nucleotides after the upstream aptamer-selection process (i.e., downstream truncation). There are several reasons for downstream truncation. First, in some cases, elimination of nonessential nucleotides can increase the binding functionality of the aptamer presumably due to reduced steric hindrance (Zhou, Battig et al. 2010). Second, although the chemical synthesis of an oligonucleotide is a well-established technique, the synthesis of aptamers longer than 60 nucleotides is always more expensive and difficult to perform, and the shorter the aptamer, the more cost-effective it is. Therefore, it is important to understand how selected aptamers interact with their targets, in order to elucidate the redundant motifs in the aptamer sequence.

As a starting point for the elucidation of the optimal structure there are several tools that can assist in identifying the essential parts of the aptamer structure, including m-fold, a software that uses thermodynamic methods to predict secondary structures using mathematical algorithms. Controlling parameters such as temperature and salt conditions this software can predict secondary structures at the minimum free energy, being able to decompose the particular folding into loops and stacks. Several other thermodynamic details are also provided, including enthalpy, entropy and an estimated Tm (Zuker 2003).

Another software available that is widely used is the QGRS Mapper, which is a software program that generates information on composition and distribution of putative Quadruplex forming G-Rich Sequences.

3.2. Structure elucidation

Several reports have been published describing the use of chromatographic and mass spectroscopy techniques for the characterisation of aptamers. A recent report describes the characterisation of superparamagnetic nanoparticles conjugated to a fluorescently labelled oligodeoxyribonucleotide by ESI-MS, and analyzed by RP-HPLC chromatography (Musumeci, Oliviero et al. 2012). Another describes a method for labelling aptamers chemiluminescently with isoluminol isothiocyanate and subsequently the ILITC-labeled aptamers were characterized by HPLC-MS and purified by HPLC (Li, Deng et al. 2010). Other authors exploited the basis of RP-HPLC to develop and use quinoline derivatives to provide a general strategy to photo-regulate oligonucleotide activity with improved caging and uncaging efficiency (Li, Shi et al. 2010). Finally, other report describes the coupling of an acoustic wave biosensor with mass spectrometry, using aptamers for the detection of protein complexes (Treitz, Gronewold et al. 2008).

In recent studies Yuanboonlim et al. combined FRET with Circular Dichroism to study the K^+ effect in the stabilisation of the G-quadruplex structure of three K^+ aptamers consisting of 12, 15, and 21 nucleotides (Yuanboonlim, Siripornnoppakhun et al. 2012). Circular Dichroism is a very common technique used for characterisation of aptamers (Ponikova, Tluckova et al. 2011) and CD is a very suitable technique to complement mass spectroscopic techniques in studies of G-quadruplex structure characterisation (Smargiasso, Rosu et al. 2008; Collie, Parkinson et al. 2010). CD can also be used as complementary technique for NMR structural characterisation (Kuryavyi, Phan et al. 2010). Furthermore Nuclear Magnetic Resonance (NMR) spectroscopy had been successfully applied to solve the solution structures of a range of aptamer or ligand-aptamer complexes. As a starting point in nucleotide characterisation a monodimensional ^{1}H-NMR is taken, these 1D spectra are recorded as DPFGSE (double pulsed field gradient selective excitation) spectra for the water suppression in order to improve signal resolution. Following this techniques like COSY, TOCSY or NOESY can be used for the final structure elucidation (Mashima, Matsugami et al. 2009; Neves, Reinstein et al. 2010). Using NMR spectroscopy techniques, the structure and dynamics of aptamers can be depicted. In addition, characterization of aptamer conformational transitions and the structural changes due to the addition of cofactors, for example, ions, small molecules or even proteins, could give an insight into biomolecular reaction mechanisms [Furtig, Buck et al. 2007; Furtig, Richter et al. 2003].

Mass spectrometry is an analytical tool used for measuring the molecular mass of a large sample such as biomolecules, where molecular masses can be measured with high accuracy and can also be used as complementary technique to NMR in order to obtain structural information. The molecular masses can be measured within an accuracy of 0.01% of the total molecular mass of the sample. That high accuracy is

sufficient to allow minor mass changes to be detected, allowing the exact mass calculation of oligonucleotides.

Furthermore structural information can be generated using certain types of mass spectrometers, like the Synapt HDMS (Waters) instrument which has a m/z 4000 quadrupole and an Ultimate 3000 HPLC system (Dionex). The Synapt HDMS instruments with quadrupole-orthogonal acceleration time-of-flight geometry have an in-built travelling wave ion mobility spectrometry device for the separation of ions with the same mass, or m/z ratio, but different cross-sectional areas which is useful for oligonucleotide sequencing (Bunka, Mantle et al. 2007).

3.3. Analytical applications of aptamers

Aptamers have been applied in a wide range of Affinity Amplification Assays. Since their appearance, aptamers have shown great promise as biocomponents for analysis not only due to their high affinity and specificity but also due to their increased stability, flexibility and versatility as compared to antibodies. Techniques involving antibodies or phage displayed antibody fragments are increasingly being replaced by aptamers in different configurations, taking advantage of the unique properties of aptamers.

Ellington was the first to exploit aptamers for the quantification of a specific protein in a cell extract using radio-labelled aptamers in a filter binding assay (Conrad and Ellington 1996). By means of previously selected RNA aptamers immobilised on a nitrocellulose filter, β II isozyme of rat protein kinase C was quantified with high reproducibility and specificity in the presence of rat brain extract. While Ellington was demonstrating the concept, at Larry Gold's NeXus Pharmaceutical Inc., Drolet et al. reported the first use of aptamers in an enzyme-linked immuno-sorbent assay (ELISA)-like assay, referred to as ELONA – Enzyme-Linked Oligo-Nucleotide Assay - or more correctly RLAA – Reporter Linker Aptamer Assay. In this first reported RLAA (Drolet, MoonMcDermott et al. 1996) the reporting antibody of a sandwich ELISA was substituted by a fluorescein-tagged RNA aptamer to detect the vesicular endothelial growth factor (VEGF) in serum. A monoclonal antibody specific for VEGF was immobilised and used to capture the target, followed by incubation of a VEGF-binding fluorescein-labelled RNA aptamer with the detection facilitated by an enzyme-labelled anti-fluorescein antibody. This cumbersome assay yielded results very similar to those obtained in typical ELISA, being able to detect concentrations down to 1 pM and without showing any cross-reactivity towards other cytokines. Although this work did not exploit specific properties of aptamers, it did highlight the possibility of aptamers to compete with, and complete the use of, antibodies in bioanalysis, paving the way for a new approach for detection. Magnetic beads (Mb) have also been exploited as supports for RLAA formats, using either immobilised or labelled aptamers as capture or detection reagents. Bruno and Kiel used magnetic beads to develop a RLAA sandwich able to detect non-pathogenic Sterne strain Bacillus anthracis spores (Bruno and Kiel

1999), cholera whole toxin and Staphylococcal enterotoxin B at nanogram to low picogram levels (Bruno and Kiel 2002). The approach used relies on a selected pool of DNA aptamer immobilised on tosyl-activated magnetic beads used to capture the analyte in solution. The detection was accomplished by exposing the beads to a second biotinylated aptamer pool followed by addition of either streptavidin-conjugated ruthenium-trisbypyridine or avidin-HRP conjugate. Rye and Nustad developed a hybrid immuno-beads assay based on 5'-biotinylated DNA thrombin aptamer and anti-thrombin antibody (Rye and Nustad 2001). In this case, sheep anti-mouse IgG was conjugated to magnetic beads and used to bind the IgG anti-thrombin monoclonal antibody. The modified beads were then exposed to a pre-incubated mixture of thrombin and biotinylated thrombin aptamer. Finally, europium (Eu)-labelled streptavidin was added for detection. The results revealed the TBA could bind the target under both stringent conditions and physiological concentrations, again highlighting the enhanced flexibility of aptamers for analytical applications.

Demonstrating the impressive robustness of their use, aptamers have been tested in depth in many affinity assay formats: besides RLAAs, aptamers has been exploited in flow cytometry, affinity chromatography and capillary electrophoresis, amongst others. In flow cytometry analysis, labelled aptamers were used for detecting (and isolating) analytes immobilised on beads (Davis, Abrams et al. 1996), as well as biomarkers expressed on cell surfaces (Davis, Lin et al. 1998; Herr, Smith et al. 2006; Shangguan, Li et al. 2006). Fluorescent aptamers have also been used in capillary electrophoresis to detect IgE in buffer and serum samples (German, Buchanan et al. 1998; Buchanan, Jameson et al. 2003) or the reverse transcriptase of the Human Immunodeficiency Virus type 1 (HIV-1) (Pavski and Le 2001; Fu, Guthrie et al. 2006). Furthermore, the high affinity, stability and the small size of the aptamers have facilitated their use in affinity chromatography. Aptamers immobilised in the stationary phase were demonstrated to obtain a very high selectivity in the retention of targets, as demonstrated by the efficient separation of arginine enantiomers (Geiger, Burgstaller et al. 1996), the purification of fusion protein from cell lysate (Romig, Bell et al. 1999) or the separation of adenosine at different phosphorylation levels (Deng, German et al. 2001).

The predisposition of nucleic acid aptamers to an assay format that combines the selectivity of the aptamers with the efficiency of nucleic acid amplification techniques, producing impressive signal enhancement with considerably lower detection limits, is exploited in apta-PCR. The first report integrating the sensitivity of nucleic acid amplification with an immunoassay, in a technique known as Immuno-Polymerase Chain Reaction (Immuno-PCR) was in 1992 (Sano, Smith et al. 1992). The use of a reporting antibody labelled with DNA, either directly, or via a biotin-streptavidin bridge, improved the sensitivity of a conventional immunoassay and enhanced the detection limits up to 100000-fold (Mweene, Ito et al. 1996; Nam, Stoeva et al. 2004; Adler 2005; Niemeyer, Adler et al. 2005). However, the technique suffers some important drawbacks, such as difficulties in labelling the antibody with nucleic acids and, furthermore, this linkage, either directly to antibodies or via biotin-streptavidin linker bridges is prone to a lack of precision often resulting in uneven numbers of oligos per antibody, resulting in high rates of error and affecting sensitivity (Niemeyer, Adler et al. 2005; McKie, Samuel et al. 2002). Additionally, following the immunorecognition step, the DNA needs to be separated from the antibody for subsequent amplification.

Aptamers on the other hand do not require to be conjugated to a label as they can inherently act both as detecting and reporting molecule, simply by flanking the aptamer with two primer sequences, thus bypassing the problems with immuno-PCR. There are several different formats described in literature, where different sandwich formats and a variety of amplification methods have been reported.

Fisher et al. demonstrated detection of thrombin via Rolling Cycle Amplification (RCA) and real time quantitative PCR (qPCR) (Fischer, Tok et al. 2008). By means of different concentrations of thrombin modified magnetic micro-particles to bind the TBA flanked by two primer regions for amplification, the group achieved a 2nM detection limit using the Rolling Cycle Amplification (RCA) and as low as a few hundred fM using qPCR. Lee and co-workers described an antibody/aptamer mixed sandwich to detect 10 *E. coli* cells/mL via qPCR (Lee, Kim et al. 2009). In an interesting report, Waga's team achieved the selection of an RNA aptamer against the constant region (Fc) of rabbit IgG (Yoshida, Sakai et al. 2008) to be used as a reporter molecule that could be taken advantage of in a multitude of assays where rabbit IgG antibodies were used. Detection was achieved via qPCR (Yoshida, Horii et al. 2009), highlighting the immense potential of the simple combination of an aptamer with antibody in affinity amplification assays.

The approach reported by Pinto et al. exploits an immobilised thrombin-binding aptamer (TBA29), used as capture aptamer, on a streptavidin- coated microtiter plate. Consequently the captured thrombin molecules were incubated with a second thrombin-binding aptamer (TBA15). The reporter aptamer (TBA15) was modified by flanking regions to include PCR primers for consequent amplification by Real Time PCR. This reported assay allowed the simultaneous recognition and quantification of

thrombin molecules captured on solid surfaces, yielding a massive improvement (20000-fold enhancement) in terms of detection limit, when compared with methods previously described. This approach is applicable to all aptamers, independent of whether multiple aptamers exists against the target, and the technique can thus be applied to the detection of very low concentrations of target analytes (Pinto et al. 2009).

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CHAPTER 2

EXTRACTION, ISOLATION, AND CHARACTERIZATION OF GLOBULIN PROTEINS FROM *LUPINUS ALBUS*

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1. ABSTRACT

Lupin has recently been added to the list of allergens requiring mandatory advisory labeling on foodstuffs sold in the European Union, and since December 2008, all products containing even trace amounts of lupin must be labeled correctly. Lupin globulins consist of two major globulins called α-conglutin (11S and "legumin-like") and β-conglutin (7S and "vicilin-like") and another additional two globulins, γ-conglutin and δ-conglutin, which are present in lower amounts. We report on a methodology to facilitate the extraction of each of these proteins using centrifugation and isolation by anion-exchange chromatography followed by size-exclusion chromatography. The isolated subunits were characterized using reducing and non-reducing polyacrylamide gel electrophoresis, western blotting, and peptide mass fingerprinting, all of which revealed that the individual protein subunits are highly pure and can be used as immunogens for the production of antibodies specific for each of the conglutin fractions, as well as standards, and the extraction protocol can be used for the selective extraction of each of the subunits from foodstuffs, thus facilitating a highly accurate determination of the lupin concentration. Furthermore, the subunits can be used to elucidate information regarding the toxicity of each of the subunits, by looking at their interaction with the IgE antibodies found in the serum of individuals allergic to lupin, providing critical information for the definition of the requirements of analytical assays for the detection of lupin in foodstuffs.

KEYWORDS: Lupin, conglutin, food allergen, peptide mass fingerprinting, anion-exchange chromatography.

2. INTRODUCTION

Lupin is an herbaceous plant of the leguminous family, belonging to the genus *Lupinus*, which includes 450 species. Lupin seeds have been used as human food and animal feed since ancient times. There are four different species of agricultural interest, white lupin (*Lupinus albus*), blue lupin (*Lupinus angustifolius*), yellow lupin (*Lupinus luteus*), and Andean lupin (*Lupinus mutabilis*) [1]. Lupin is considered a source of low-cost protein and can be cultivated in colder climates, making it attractive in comparison to other protein-rich plants [1], and is attracting attention as an excellent food material with a high nutritional value [2-4]. Lupin or lupin flour is used in bread, cookies, pastry, pasta, and sauces and also in beverages as a substitute for milk or soy, and the seeds are widely used as snacks [5-10]. The seed storage proteins of different species have been studied in detail from the turn of the century, when Osborne in 1924 classified them into four groups on the basis of their extraction and solubility characteristics: albumins, globulins, glutenins, and prolamins [11,12]. However, it is well-known that a clear-cut distinction between these groups of proteins is not always possible, with many proteins showing intermediate solubility behaviors [13]. The globulin fraction of lupin protein consists of two major globulins called α-conglutin (11S and "leguminlike"), accounting for about 33% of the total protein content in *L. albus* [14,15], and β - conglutin (7S and "vicilin-like"), which accounts for another 45% [14,16]. Finally, there are two additional globulins of minor quantity called γ- and δ-conglutin, which account for 5 and 12%, respectively, in *L. albus* [10,14,15,17]. Some reports have further sub-classified δ-conglutin into δ_1- and δ_2- conglutin (Table 2.1.) [18,19].

Table 2.1. Summary of *Lupinus albus* conglutins' main features (Duranti, Consonni et al. 2008).

Conglutin (Protein Family)	**Svedberg Velocity**	**UniProtKB TreEMBL (acc. n.)**	**(%) of globulins**	**Native protein**				**Upon reduction**	
				M_w, kDa	pI	Quaternary Structure	Subunits Size, kDa	Heavy Chain	Light Chain
δ-conglutin (sulphur rich)	2S	Q333K7	10-12	13	4.3	Monomer		9	4
α-conglutin (legumin-like)	11S	Q53I54	35-37	330-430	5.6-5.9	Hexamer	53 60 66 70	31 36 42 46	19 19 19 19
β-conglutin (Vicilin-like)	7S	Q53HY0 and Q6EBC1	44-45	143-260	5.9-6.2	Trimer	19-60	No disulphide bonds	
γ-conglutin	7S	Q9FEX1 and Q9FSH9	4-5	200	7.9	Tetramer	47	29	17

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Lupin, in the form of flour, seed, or dust, has been reported to produce a variety of different allergic responses, such as urticaria and angioedema [20,21], contact urticarial [22], oral allergy syndrome [1], rhino conjunctivitis [21,23], and anaphylaxis [1,24-27]. Lupin allergy apparently arises by either primary sensitization [1,23,28] or clinical cross-reactivity in individuals who are allergic to peanut [15,21,29]. These cross-allergic clinical reactions of peanut to other members of the leguminous family, such as soy, peas, beans, and lentils, occurs in about 5% of peanut-allergic patients but were found to be 68% with lupin [29]. Because lupin was officially admitted as a food ingredient in France in 1997, a high number of severe food allergy reactions to lupin have been reported. In the work reported here, a procedure for lupin protein extraction, isolation, and characterization of lupin proteins is optimized, to obtain pure targets that can be used as standards, immunogens for specific antibody production, and can also find application in human toxicity studies.

3. MATERIALS AND METHODS

Reagents. Buffers, acids or bases, and salts were purchased from Sigma (Barcelona, Spain), and the electrophoresis reagents, staining solutions, and standards were purchased from Bio-Rad (Barcelona, Spain). Columns and chromatographic matrices were purchased in General Electric Healthcare (Barcelona, Spain). Trypsin was purchased from Roche Molecular Biochemicals (Barcelona, Spain), and the mass spectra standard calibrator kit was purchased from per septive Biosystems (Barcelona, Spain).

Instrumentation. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analyses were performed on a Voyager-DE STR instrument (PerSeptive Biosystems, Framingham, MA).

Plant Material. Dry seeds of white lupin (*L. albus*) were used. The dry cotyledons were milled (0.2 mm sieve) with a grinder (Moulinex, Moulinette). The resulting flour was defatted with n-hexane (35 mL/g of dry weight) under vigorous vortexing for 10 min at room temperature (RT). The defatted flour was centrifuged at 6000 g for 10 min at RT, and the pellet obtained was dried under vacuum for 72 h, after decantation of n-hexane.

Extraction of Globulins with Dilute Salt Solutions. A diagram providing an overview of the extraction protocol can be seen in Figure 2.1. The albumin fraction and other minor soluble constituents were extracted with water (adjusted to pH 8) containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$ under vigorous vortexing for 10 min at RT. The suspension was centrifuged at 30000g for 15 min at 4 ºC, and the supernatant containing the albumin fraction was collected. To minimize the residual albumin content present in the pellet, a second albumin extraction was performed and the supernatant was removed. The globulin fraction was extracted from the pellet with 100 mM Tris-HCl (pH 7.5) containing 10% (w/v) NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM ethylene glycol bis (R-aminoethyl ether)-N,N,N0,N0-tetraacetic acid (EGTA), by vigorous vortexing for 10 min at RT. The globulin-containing solution was centrifuged at 30000g for 15 min at 4 ºC, and the supernatant obtained was dialyzed against phosphate-buffered saline (PBS) (pH 7.6) and stored at 4 ºC [10].

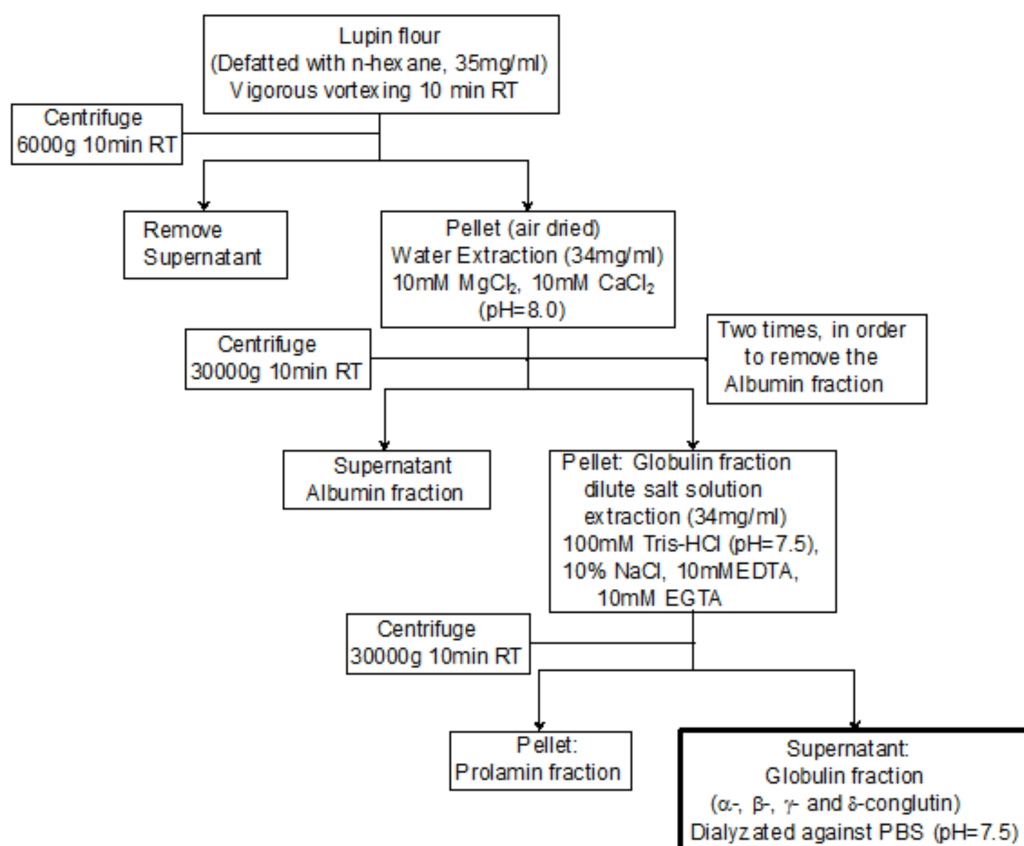

Figure 2.1. Scheme of the Extraction Protocol for Globulins with Dilute Salt Solutions.

Anion-Exchange Chromatography (AEC). The different globulin fractions were isolated by AEC on a HiTrap ANX fast flow (FF) ion-exchange pre-packed column linked to a peristaltic pump. The column was equilibrated with 50 mM Tris-HCl (pH 7.5), loaded with 10 mL of the globulin fraction, and eluted with a linear gradient elution at a mobile phase flow of 0.5 mL/min, and the absorbance of the fractions was measured at 280 nm of wavelength. The sample (10 mL) was added followed by a washing step of 20 min, and the gradient was established by increasing the concentration of NaCl every 2 min in steps of 0.01 M from 0 to 1 M. Under these conditions, γ-conglutin was not retained on the column and was immediately eluted. Fractions were eluted from 0 to 0.7 M NaCl, and the salt concentrations for elution of specific β-conglutin and combined α- and δ-conglutin fractions were optimized to be 0.3 and 0.45 M, respectively (Figure 2.2.). Fractions of the isolated subunits were lyophilized with a Freezone 1 Labconco apparatus at 0.066 mbar of vacuum and -50 ºC. After lyophilization, the fractions were redissolved in 50 mM Tris-HCl (pH 7.5) and stored at 4 ºC.

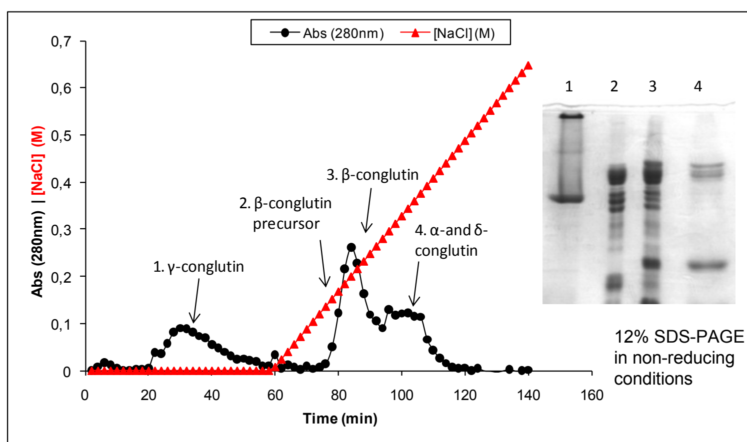

Figure 2.2. Anion exchange chromatography. Medium: ANX Sepharose 4 Fast Flow; Bead size: 45-165 µm; Type of gel: Weak anion; Charged group: -N+(C2H5)2H; Sample: globulin extract; Sample Volume: 10 mL (1.7mg/mL); Buffer: 50 mM Tris HCl, pH = 7.5, Fractions were eluted from 0-0.7 M NaCl at intervals of 16 mM; Flow: 0.5 mL/min. NOTE: The units of absorbance at 280 nm and the concentration of sodium chloride coincide in the y-axis.

Size-Exclusion Chromatography (SEC) by Sephacryl-200 HR. α-Conglutin was purified from δ-conglutin by SEC using a self-prepared column (0.8 cm in diameter and 9 cm in height), packed with Sephacryl-200 HR and linked to a peristaltic pump. The column was equilibrated with 15 mM NaCl in 50 mM Tris-HCl (pH 7.5) and loaded with 0.2 mL of the combined α- and δ-conglutin fraction. The mobile phase flow was 0.1 mL/min. The fractions were collected every 2 min, and the absorbance of the fractions was measured at 280 nm.

Sodium Dodecyl Sulfate-Polyacrilamide Gel Electrophoresis (SDS-PAGE). Proteins from extracts were separated on SDS-PAGE (12% resolving gel and 4% stacking gel) performed in avertical electrophoresis unit at an applied voltage of 70 V. When the tracking dye migrated to the bottom of the stacking gel, the voltage was then increased to 120 V until the tracking dye migrated to the bottom edge of the resolving gel. Gels were stained with Coomassie Brilliant Blue R-250 (0.05%, w/v) in methanol/acetic acid/water (25:10:65, v/v/v) and destained in the same solution without the dye.

Western Blotting. Proteins separated by 12% SDS-PAGE in reducing conditions were electroblotted onto a Immobilon-P membrane according to the method by Towbin et al. [30] on a Trans-blot electrophoretic transfer cell. The membranes were blocked in 5% bovine serum albumin (BSA) and washed 3 times in TBS-Tween at pH 7.5. Membranes were then soaked for 1 h at RT with 10% biotinylated rabbit antilupin antibody from the HAVen lupin enzyme-linked immunosorbent assay (ELISA) kit, diluted 1:100 000 in 20 mM Tris-HCl buffer at pH 7.5 with 150 mM NaCl,1 containing 1% (w/v) BSA, 0.5% (w/v) powdered milk, and 0.1% powdered sodium azide, and followed by the addition of streptavidin-horseradish peroxidase (HRP) (10 µg/mL) for 15 min at RT. Finally, 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate was added until the precipitate was formed on the membrane for visualization.

Mass Spectrometric Analysis. To further characterize the isolated globulin proteins, peptide mass fingerprinting was performed for all of the bands obtained in the SDS-PAGE. Electrophoresis bands obtained were cut from the gel, and the proteins eluted. The isolated proteins were digested with trypsin, and the resulting peptide mixture, extracted from the polyacrylamide gel, was applied to MALDI-TOF and, finally, analyzed by MS, generating a peptide mass profile characteristic for the excised protein.

In-Gel Destaining, Reduction, Alkylation, Deglycosylation, and Digestion of Protein Samples. The bands of interest were manually excised from preparative Coomassie-stained 1-DE gels, depending upon their intensity and relative volume, destained by washing twice with 50% acetonitrile in 25 mM ammonium bicarbonate, and vacuum-dried. Bands were reduced with 10 mM dithiothreitol in 25 mM ammonium bicarbonate for 30 min at 56 ºC and, subsequently, alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 20 min in the dark. Gel pieces were alternately washed with 25 mM ammonium bicarbonate and acetonitrile and dried under vacuum. Gel bands were subsequently shrunk with acetonitrile and vacuum-dried. All gel pieces were incubated with 12.5 ng/µL sequencing-grade trypsin in 25 mM ammonium bicarbonate overnight at 37 ºC. After digestion, the supernatants (crude extracts) were separated. Peptides were extracted from the gel pieces first into 50% acetonitrile and 1% trifluoroacetic acid and then into 100% acetonitrile. All extracts were pooled, and the volume was reduced by SpeedVac [31].

MALDI-TOF MS. A total of 1 µL of each sample (extracted peptides) and then 0.4 µL of 3 mg/mL α-cyano-4-hydroxycinnamic acid matrix (Sigma) in 50% acetonitrile and 0.01% trifluoroacetic acid were spotted onto a MALDI target. Samples for MS were mixed 1:1 with α-cyano matrix (5 mg/mL in 50% acetonitrile and 0.3% trifluoroacetic acid) and spotted on the stained steel plate. Peptides were selected in the mass range of 750-3500 Da. All mass spectra were calibrated externally with the Sequazyme peptide mass standard kit and internally with trypsin autolysis peaks [31].

4. RESULTS AND DISCUSSION

Extraction of Globulins and Isolation of Conglutin Subunits. The protein extracts obtained showed the expected electrophoresis pattern (results not shown) for isolated, purified globulins, containing all of the α-, β-, γ-, and δ-conglutins. The purified globulin fraction was then applied to AEC for isolation of each of the globulin components. As previously reported by Dooper et al. [10] using AEC, the γ-conglutin is not retained on the column, the β-conglutin precursor and β-conglutin are eluted by a linear gradient of NaCl at 0.3 M, and at 0.45 M NaCl, the α- and δ-conglutins are co-eluted (Figure 2.2.). As can be seen in lane 1 of Figure 2.3.a, the electrophoresis pattern of the non-retained fraction of the AEC performed showed the expected γ -conglutin electrophoresis pattern, with a band obtained at 43 kDa of γ-conglutin, which, as can be seen in lane 1 of Figure 2.3.b, upon reduction, is divided into its two subunits of 30 and 17 kDa. For the first eluted fractions at a concentration of 0.35 M NaCl, the bands obtained are indicative of β -conglutin (lane 3) and its precursor, obtained at a concentration of 0.25 M NaCl (lane 2) with bands ranging from 19 to 60 kDa, corresponding to the noncovalently linked subunits. The identity of the components was confirmed using the peptide mass fingerprinting. The expected α- and δ-conglutin electrophoresis pattern that was obtained in the eluted fraction at 0.45 M NaCl in 50 mM Tris-HCl (pH 7.5) shows bands at 70, 66, 60, and 53 kDa (lane 4 of Figure 2.3.a), which, upon reduction, are split in the heavy chain of a size of 46, 42, 36, and 31 kDa, respectively, and light chain of 19 kDa (lane 4 of Figure 2.3.b). A band of approximately 20 kDa and less than 15 kDa that can be ascribed to δ-conglutin is also observed under both reducing and nonreducing conditions.

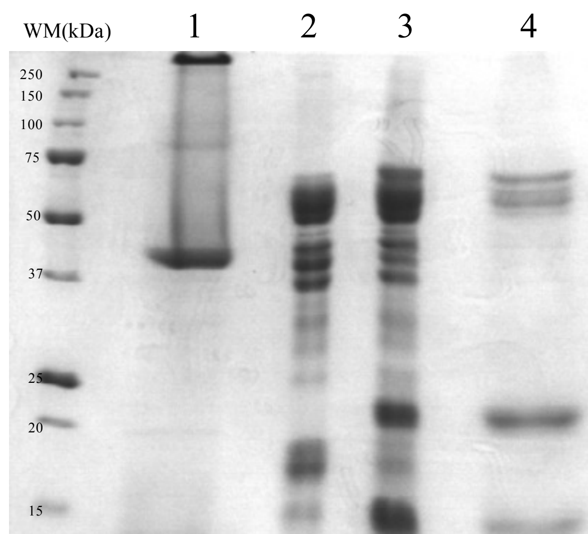

Figure 2.3.a. Non-reducing conditions SDS-PAGE of the fractions obtained. WM: weight marker; Lane 1: γ- conglutin; Lane 2: β- conglutin precursor; Lane 3: β-conglutin; Lane 4: α- conglutin and δ-conglutin.

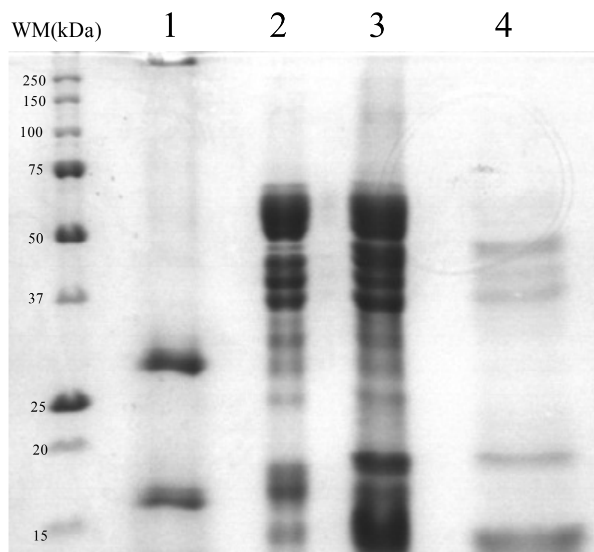

Figure 2.3.b. Reducing conditions SDS-PAGE of the fractions obtained. WM: Weight marker (kDa); Lane 1: γ- conglutin; Lane 2: β- conglutin precursor; Lane 3: β-conglutin; Lane 4: α- conglutin and δ - conglutin.

The isolation of α-conglutin from δ-conglutin was not possible in a single purification step using AEC, and the most notable difference between these globulins is their molecular weight, being 330-430 and 13 kDa, respectively (Table 2.1.). SEC was chosen as the most appropriate technique to obtain pure proteins. As seen in Figure 2.4, a clear isolation of the two proteins were obtained, and Figure 2.5 shows the purity of each of the isolated proteins, among others.

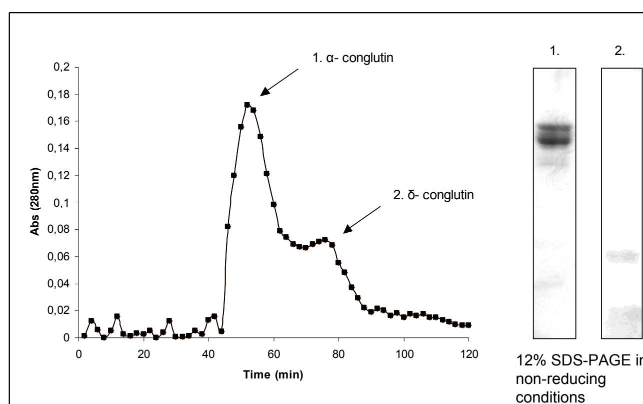

Figure 2.4. Size exclusion chromatography. Medium: Sephacryl S-200 HR; Sample: globulin fraction previously eluted at 1 M NaCl by AEC; Sample Volume: 0,2 mL (1.34 mg/mL); Column packed bed: 0.8 x 9 cm; Buffer: 50 mM Tris HCl, pH=7.5, 0.15 M NaCl; Flow: 0.1 mL/min.

The protein bands were then analyzed using peptide mass fingerprinting, and the profile obtained for each band was then compared to the theoretical masses derived from the digestion in silico at the same enzyme excising sites of all protein sequences of both the Swissprot and Genbank databases. The proteins in the database were then ranked using peptide mass fingerprinting according to the number of peptide masses matching their sequence, within a given error tolerance in mass [31]. The same procedure was applied to all of the bands obtained under both non-reducing and reducing conditions and positively confirms the isolation of the γ-conglutin, β-conglutin precursor, β -conglutin, and δ -conglutin subfractions, with very good sequence coverage and very high score (see the Supporting Information for detailed PMF analysis). It was possible to positively confirm the α-conglutin by PMF because it is only possible to find one fragment of this component in the databases coming from *L. angustifolius* [accession number of the National Center for Biotechnology Information (NCBI) is AAC49787, described as conglutin R (*L. angustifolius*) gi|2313076|gb|AAC49787.1|(2313076)] and one complete amino acid sequence for *L. albus* (accession number of UniProtKB/TrEMBL is Q53I54). However, correlating this fragment and amino acid sequence and taking into consideration the electrophoresis

pattern and the mass spectra profile obtained, it can be proposed that it is isolated α-conglutin.

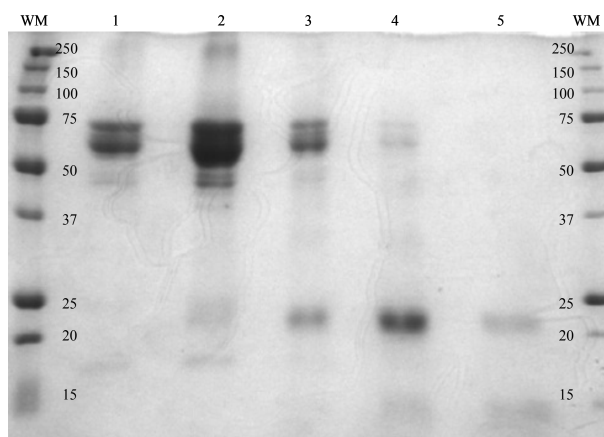

Figure 2.5. 12% SDS-PAGE in non-reducing conditions of the fractions obtained. WM: weight marker; Lane 1: Fraction 23-25, α- conglutin isolated; Lane 2: Fraction 26-29; Lane 3: Fraction 30-32; Lane 4: Fraction 33-36; Lane 4: Fraction 37-44, δ- conglutin isolated.

Western Blotting Analysis. As outlined in the Introduction, in individuals with lupin allergy, the globulin fractions have been suggested to be important allergens [32]. Furthermore, lupin has recently been added to the list of allergens requiring mandatory advisory labeling on foodstuffs sold in the European Union; thus, all products containing even trace amounts of lupin must be labeled correctly since December 2008 according to the European Commission Directive 2006/142/EC [European Commission (EC)] Annex IIIA of Directive 2000/13/EC [33], in response to the increasing number of severe cases of lupin allergies reported during the past decade [34].

Many studies have been carried out to identify which specific lupin fraction(s) are responsible for provoking the allergenic immune response. Initial studies outlining the isolation and characterization of protein bodies in *L. angustifolius* reported that γ - conglutin reacted with monoclonal antibodies, but the 2S sulfur-rich storage protein (δ-conglutin) did not produce any reaction [35]. Other studies, focused more on allergy to lupin flour, reported several IgE-binding proteins. The analysis of the serum of a patient who died after eating hot dog bread showed a high anti-peanut IgE level. Proteins with 38 and 15 kDa were responsible for the cross-reaction with the anti-peanut IgE of the patient, and a polyclonal anti-lupin serum detected three main protein bands at 65, 50, and 40 kDa in all extracts containing lupin. A cross-reaction against peanut proteins was negligible at high dilutions [29].

More recent studies reported since 2005 maintain that γ-conglutin is the major allergen of lupin and that γ-conglutin polypeptides were also found to cross-react with IgG specific to the basic subunit of Ara h 3, a known peanut allergen of the 11S globulin family. This polypeptide of 43 kDa, which might coincide with the unreduced γ-conglutin, was also observed to produce cross-reactivity with anti-peanut IgE. The latest publications indicate that all of the α-, β-, γ-, and δ-conglutin subunits are candidate allergens and suggest a particularly strong allergenicity of α- and γ-conglutins. Furthermore, the results indicate the presence of cross-reactive allergens in lupin, peanut, and almond [36,37].

A specific and sensitive analytical method for detecting and quantifying each one of the lupin conglutin protein is thus required for the protection of the consumers with food allergy from the danger of hidden allergens. The *L. angustifolius* conglutin β allergen has recently been designated Lup an 1 by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-committee [38]. However, as detailed above, there are reports indicating that all four subunits can provoke a toxic response. Currently, there are few commercial ELISA kits available on the market to detect lupin allergens [1,10,39,40]; however, these kits are not specific for each of the subunits, and none of them are capable of detecting the γ- and δ-conglutins. A realtime polymerase chain reaction (PCR) has also been used for the detection of lupin, increasing the sensitivity [41-43] but still incapable of specifically detecting each subunit, except for one report detailing the specific detection of γ- and δ-conglutins as target immunogens [43].

Using commercially available antibodies from the HAVen lupin ELISA kit, western blotting analysis was carried out, and as can be seen in Figure 2.6., these antibodies interact with the β-conglutin precursor, as well as both of the α- and β-conglutin subunits, whereas no interaction with the δ- or γ-conglutin proteins is observed. These results can be expected because the antibodies used in the kit were produced using a mixture of uncharacterized β-, α-, and δ-conglutin proteins as the target immunogen. These results confirm the isolation of the α protein from the δ protein but also highlight that the kit does not detect δ- or γ-conglutin protein, despite the above-mentioned reports that the γ-conglutin protein also provokes toxicity, and furthermore, the assay detects the γ-conglutin protein, even though there are reports that it does not provoke toxicity. It is clear that there is a definite requirement for an analytical assay that specifically detects each of the proteins [38,44,45].

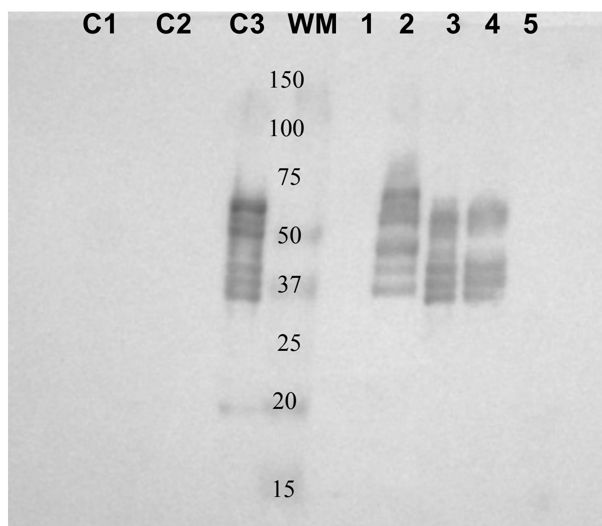

Figure 2.6. Western blot of the fractions obtained. Lane 1: δ- conglutin; Lane 2: α-conglutin; Lane 3: β- conglutin; Lane 4: β- conglutin precursor; Lane 5: γ- conglutin; WM: Kaleidoscope weight marker (8 μL) Bio-Rad; Lane C1: PSA; Lane C2: BSA; Lane C3: Crude extract.

In conclusion, in this work, we report on the characterization of conglutin proteins isolated from lupin globulins. The isolated fractions were characterized using reducing and non-reducing electrophoresis, western blotting, and peptide mass fingerprinting, all of which confirmed the purity of each of the conglutin subunits. Furthermore, the peptide mass fingerprinting results provided new mass spectra for α-conglutin for a protein sequence not introduced in the Swissprot and Genbank databases.

These isolated pure proteins can be further used as immunogens for raising specific antibodies, as well as standards for the analytical detection of specific proteins. The protocol elucidated can also be used to selectively extract and purify specific globulin proteins from commercial foodstuffs for analysis. Furthermore, the isolated proteins can be used to elucidate information regarding toxicity of each protein by looking at their interaction with the IgE antibodies found in the serum of individuals allergic to lupin, such as reported by Goggin et al. [38], who reported possible contamination of their isolated α-conglutin with β-conglutin, giving rise to a false-positive result, and the authors also indicate that the toxicity of the γ-conglutin needs to be further investigated.

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6. SUPPORTING INFORMATION

Table 2.S1. Peptide Mass Fingerprinting of protein isolates. Bands from lanes of non reducing conditions SDS-PAGE.

Lane	SDS-PAGE Mass (kDa)	Protein name	Species	Accession number	Mass	pI	Sequence Coverage	Peaks Matched	Peaks Matched (%)
1	250	γ-conglutin	*Lupinus albus*	11191819	49219	8.4	26.5	9/57	16
1	40	γ-conglutin	*Lupinus albus*	11191819	49219	8.4	31.6	9/46	20
3	63	β-conglutin precursor	*Lupinus albus*	46451223	62092	6.43	47	24/59	41
3	61	β-conglutin	*Lupinus albus*	89994190	61994	6.08	47	28/45	47
3	51	β-conglutin (Fragment)	*Lupinus albus*	Q53HY0_LUPAL	61994	6.08	45	27/56	48
3	49	β-conglutin	*Lupinus albus*	89994190	61994	6.08	43	26/70	37
3	46	β-conglutin precursor	*Lupinus albus*	46451223	62092	6.43	34	17/51	33
3	44	β-conglutin	*Lupinus Albus*	89994190	61994	6.08	37	22/52	42
3	38	β-conglutin	*Lupinus albus*	89994190	61994	6.08	38	20/65	31
3	35	β-conglutin	*Lupinus albus*	89994190	61994	6.08	33	19/80	24
3	31	β-conglutin	*Lupinus albus*	89994190	61994	6.08	32	20/102	20
4	63	β-conglutin	*Lupinus albus*	89994190	61994	6.08	49	28/78	36
4	62	β-conglutin	*Lupinus albus*	89994190	61994	6.08	46	28/52	54
4	61	β-conglutin	*Lupinus albus*	89994190	61994	6.08	47	26/49	53
4	51	β-conglutin	*Lupinus albus*	89994190	61994	6.08	45	25/49	51
4	49	β-conglutin	*Lupinus albus*	89994190	61994	6.08	48	28/54	52
4	46	β-conglutin	*Lupinus albus*	89994190	61994	6.08	44	25/61	41
4	44	β-conglutin	*Lupinus albus*	89994190	61994	6.08	37	24/52	46
4	38	β-conglutin	*Lupinus albus*	89994190	61994	6.08	37	23/54	43
4	35	β-conglutin	*Lupinus albus*	89994190	61994	6.08	38	22/43	51
4	31	β-conglutin	*Lupinus albus*	89994190	61994	6.08	35	21/53	40
4	28	β-conglutin	*Lupinus albus*	89994190	61994	6.08	49	28/78	36
5	66	β-conglutin	*Lupinus albus*	Q53HY0	62032	6.1	25	9/42	21
5	60	β-conglutin	*Lupinus albus*	Q53HY0	62032	6.1	51	28/78	35
5	53	β-conglutin	*Lupinus albus*	89994190	62032	6.1	34	16/62	25
5	19	δ-conglutin	*Lupinus albus*	Q333K7	17138	5.5	61	8/25	32
C+	70	albumin	*Bos taurus*	30794280	71274	5.82	42	24/46	52
C+	25	kallikrein 3, (PSA)	*Homo sapiens*	119592329	24056	6.82	46	10/57	18
C+	69	Chain A, HSA	*Homo sapiens*	3212456	68425	5.67	35	24/58	41
C-	-	-	-	-	-	-	-	No Peaks	No Peaks

Table 2.S2. Peptide Mass Fingerprinting of protein isolates. . Bands from lanes of reducing conditions SDS-PAGE

Lane	SDS-PAGE Mass (kDa)	Protein name	Species	Accession number	Mass	pI	Sequence Coverage	Peaks Matched	Peaks Matched (%)
1	30	γ-conglutin	*Lupinus albus*	11191819	49219	8.4	14	5/38	13
1	17	γ-conglutin	*Lupinus albus*	11191819	49219	8.4	26	7/37	19
3	63	β-conglutin precursor	*Lupinus albus*	46451223	62130	6.4	55	29/55	52
3	61	β-conglutin	*Lupinus albus*	89994190	62032	6.1	53	30/47	63
4	61	β-conglutin	*Lupinus albus*	89994190	62032	6.1	60	32/53	60
4	38	β-conglutin	*Lupinus albus*	89994190	62032	6.1	61	32/59	54
C+	25	PSA precursor	*Human*	P07288	28742	7.6	38	10/32	31
C-	-	-	–	–	–	–	–	No Peaks	No Peaks

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CHAPTER 3

DNA APTAMERS AGAINST THE LUP AN 1 FOOD ALLERGEN

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1. ABSTRACT

Using in vitro selection, high affinity DNA aptamers to the food allergen Lup an 1, β-conglutin, were selected from a pool of DNA, 93 bases in length, containing a randomised sequence of 49 bases. β-conglutin was purified from lupin flour and chemically crosslinked to carboxylated magnetic beads. Peptide mass fingerprinting was used to confirm the presence of the β-conglutin. Single stranded DNA was generated from the randomised pool using T7 Gene 6 Exonuclease and was subsequently incubated with the magnetic beads and the captured DNA was released and amplified prior to a further round of Systematic Evolution of Ligands by Exponential Enrichment (SELEX). Evolution was monitored using enzyme linked oligonucleotide assay and surface plasmon resonance. Once a plateau in evolution was reached, the isolated DNA sequences were cloned and sequenced. The consensus motif was identified via alignment of the sequences and the affinities of these sequences for immobilised β-conglutin were determined using surface plasmon resonance. The selected aptamer was demonstrated to be highly specific, showing no cross-reactivity with other flour ingredients or with other conglutin fractions of lupin. The secondary structures of the selected aptamers were predicted using m-fold. Finally, the functionality of the selected aptamers was demonstrated using a competitive assay for the quantitative detection of β-conglutin. Future work will focus on structure elucidation and truncation of the selected sequences to generate a smaller aptamer for application to the analysis of the Lup an 1 allergen in foodstuffs.

2. INTRODUCTION

Lupin is an herbaceous plant of the leguminous family belonging to the genus *Lupinus*. Lupin seeds have been used as human food and animal feed since ancient times and is considered a low-cost protein source. It can be cultivated in cold climates, making it attractive in comparison to other protein rich plants and is an excellent food material with a high nutritional value. Lupin flour and seeds, which are widely available as snacks, are also used in bread, cookies, pastry, pasta, sauces, as well as in beverages as a substitute for milk or soy. However, in response to the increasing number of severe cases of lupin allergies reported during the last decade, in December 2008 lupin was added to the list of substances requiring mandatory advisory labelling on foodstuffs sold in the European Union [1,2]. The globulin fraction of lupin protein has been associated with its allergenicity and consists of two major globulins called α-conglutin (11S and “legumin-like”), accounting for about 33% of the total protein content in *L. albus*, and β-conglutin (7S and “vicilin-like”), which accounts for another 45%. There are two other globulins, γ- and δ-conglutin, which account for 5 and 12%, respectively, and there have been reports that these fractions are also responsible for allergenicity. Thus, all products containing even trace amounts of lupin must be labelled correctly [3] and the International Union of Immunological Societies (IUIS) allergen nomenclature subcommittee recently designated β-conglutin as the Lup an 1 allergen [4]. Currently available commerial enzyme linked immunosorbent assays exploit polyclonal antibodies that are not specific to β-conglutin [5] and reports in the literature only detail monoclonal IgG antibodies against α-conglutin and IgM antibodies against β-conglutin [6,7], and there are no reports or commercial ELISAs for the specific detection of β-conglutin. There is thus a need for an analytical tool/method that can specifically detect the Lup an 1 allergen, β-conglutin.

In the middle of the 19th century Darwin outlined one of the most studied mechanisms of evolution, which he termed natural selection [8], and a century later the first evolutionary experiments with nucleic acids were carried out by Spiegelman using an RNA-dependent RNA replicase for the replication of RNA species [9-12]. In the beginning of 1990’s an artificial in vitro selection technique for the isolation of specific nucleic acid sequences was reported by three laboratories [13-15]. The technique used was termed SELEX, which is an acronym for Systematic Evolution of Ligands by Exponential Enrichment [15], and is a procedure in which a large population of random sequences in a synthetically produced library of nucleic acids is used to select specific aptamers by iterative rounds of systematic binding, competition, selection, amplification, and enrichment. The target-aptamer interaction is normally due to the formation of secondary structures that may act as a scaffold for the target [16].

These selected nucleic acid sequences are called aptamers derived from the Latin *aptus*, "to fit" and the Greek word *meros* "part", and are artificial ligands [13], with the ability to bind to non-nucleic acid target molecules ranging from large complex molecules such as protein [15, 17-19] to simple organic small molecules like ATP [20,21], dyes [13,22], amino acids [23,24] or simple small cations [25], with high affinity and specificity, even being capable of discriminating between enantiomers [26]. There have been reports of aptamers for food safety, capable of the detection for biotoxins such as the mycotoxins ochratoxin A [27] and fumonisin B [28] and endotoxin [29], for a range of antibiotics with detection limits ranging from nanomolar to micromolar, e.g. Kanamycin A [30] and B [31], neomycin [32], tetracycline [33], chloramphenicol [34], as well as for various bacterial pathogens, including *Salmonella typhimurium* [35], *Escherichia coli O157:H7* [36] *Listeria monocytogenes* [37] and *Staphylococcus aureus* [38]. Lysozyme is the only example of a food allergen that aptamers have been selected for and both RNA [39] and DNA [40] aptamers have been selected, with equilibrium dissociation constants of 2.8 ± 0.3 nM and 0.8 ± 2.0 nM [41], as measured by fluorescence anisotropy at 25ºC.

The objective of this work is the selection of a single stranded DNA aptamer that is specific for the Lup an 1 allergen, β-conglutin. The β-conglutin subunit from lupin was first purified and was then chemically crosslinked to magnetic beads. The protein-conjugated magnetic beads were evaluated using peptide mass fingerprinting to ensure the presence of the β-conglutin on the surface of the beads. A DNA library pool with 10^{14} population variability was amplified using primers where the forward primer was phosphorothioated and single stranded DNA was generated using the T7 Gene 6 Exonuclease yielding 93-mer DNA sequences, which were incubated with the protein-conjugated magnetic beads. Each round of SELEX was monitored using PCR, comparing the amount of DNA liberated from the protein-conjugated beads to that obtained from the unconjugated magnetic beads. Evolution was monitored using enzyme linked oligonucleotide assay (ELONA) and surface plasmon resonance (SPR) and after 15 rounds of SELEX the enriched DNA was cloned, sequenced and consensus motifs identified. The affinity and specificity of these motifs were also evaluated and their secondary structure predicted. Finally the aptamers obtained were applied in a competitive ELONA format for the detection and quantification of the β-conglutin lupin allergen.

3. MATERIALS AND METHODS

Reagents: Phosphate buffered saline (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4), PBS-tween (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4, 0.05% v/v Tween 20), 3, 3', 5, 5' tetramethyl benzidine (TMB), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC), N-Hydroxysuccinimide (NHS) and all other regents were purchased from Sigma (Barcelona, Spain). Sodium chloride, sodium hydroxide 2 M, hydrochloric acid 6 M, concentrated nitric acid, ampiciline sodium salt, LB medium and agar were purchased from Scharlau Chemie S.A. (Barcelona, Spain). Trypsin was purchased from Roche Molecular Biochemicals, and mass spectra standard calibrators kit from Per Septive Biosystems. Dynabeads® M-270 Carboxylic Acid, TOPO TA Cloning® kit, Tfi DNA Polymerase, 10 bp DNA ladder, One Shot® Top 10 Chemically competent *E. coli*, Ultrapure X-gal from invitrogen (Invitrogen, Spain). T7 Gene 6 Exonuclease was purchased in USB Corporation (Cleveland, Ohio, USA). Certified™ Low Range Ultra Agarose and Precision Plus Protein™ Standards were purchased on Bio-Rad (Barcelona, Spain). Oligonucleotides (HPLC purified and provided lyophilized) were synthesized by Ella Biotech GmbH (Martinsried, Germany). Oligonucleotides and reagents were used as purchased without further purification. All solutions were prepared in high purity water obtained from a Milli-Q RG system (Barcelona, Spain).

Preparation of protein-conjugated magnetic beads: Proteins from *Lupinus albus* seeds were extracted, purified and characterized as previously described [42], obtaining a pure isolate of β-conglutin. β-conglutin was conjugated to Dynabeads® M-270 Carboxylic Acid magnetic beads using carbodiimide coupling. Magnetic beads (100 µL, 2 × 109 beads/mL) were washed with 25 mM MES, pH 5. After washing, the solution was placed beside a magnet for 4 min and the washing solution removed. The 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC) solution (50 µL, 50 mg/mL) and N-Hydroxysuccinimide (NHS) solution (50 µL, 50 mg/mL) were then added and incubated for 30 min at room temperature under shaking conditions. Following incubation, the EDC/NHS solution was removed and the magnetic beads were washed twice with 100 µL of 25 mM MES pH 5, and incubated with the target of interest β-conglutin (100 µL, 2 µg/µL) overnight at room temperature under shaking conditions. After incubation, the mixture of magnetic beads and target was placed on the magnet for 4 min and any unbound β-conglutin was removed. To block unreacted carboxylic groups on the magnetic bead surface 100 µL of 50 mM ethanolamine in PBS (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 8.0) was added and incubated at room temperature under shaking conditions for 1 hour. After incubation, the solution was again placed in contact with the magnet for 4 min and the ethanolamine solution was removed by washing three times with 100 µL of PBS-tween pH 8 and the β-conglutin conjugated magnetic beads were resuspended in 100 µL of PBS (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4).

Characterization of protein-conjugated magnetic beads: 10 µL of the magnetic beads suspension were washed two times with 50 µL ammonium bicarbonate 25 mM, pH 8 and the supernatant was removed after 2 min of incubation, followed by addition of 10 µL of 20 mM DTT in 50 mM ammonium bicarbonate and incubation for 1 h at 56ºC. The supernatant was again removed via magnetic separation, and 10 µL of 50 mM iodoacetamide in 50 mM ammonium bicarbonate added and incubated for 20 min at 21ºC, protected from light, followed by supernatant removal. Following the reduction and alkylation steps, the proteins on the magnetic bead surface were digested using trypsin in 25 mM ammonium bicarbonate, in a protein/trypsin ratio (w/w) of 1/50, for 16 hours at 37ºC prior to sonication for 10 min at 4ºC. One microliter of each sample of extracted peptides was spotted onto a MALDI plate, and when it was almost dry 1 µL of the matrix was added (3 mg/mL α-cyano-4-hydroxycinnamic acid matrix in 50% acetonitrile, 0.1% trifluoroacetic acid). Peptides were selected in the mass range of 750–3500 Da, and acquired in the positive reflector mode. All mass spectra were externally calibrated with the Sequazyme peptide mass standards kit and internally with trypsin autolysis peaks, and processed using Data Explorer Software and MASCOT for matching the spectra profile obtained with the NCBI/UniProtKB/TrEMBL database.

In vitro Selection: SELEX. The DNA library pool used consisted of diverse 93-mer DNA sequences containing a random region of 49 nucleotides flanked by primer regions as a template SLAu: 5'-agc tga cac agc agg ttg gtg n49ca cga gtc gag caa tct cga aat-3', the forward Primer SLFAuPTO: 5'-a*g *c* t*g *ac aca gca ggt tgg tg-3', * corresponds to a phosphorothioate and the reverse primer SLRAu: 5'-att tcg aga ttg ctc gac tcg tg-3'. In the first two rounds the DNA library pool (0.5 nmol) was denatured at 95ºC for 4 min and then allowed to cool at 4ºC for 10 min, after which 5 µL of positive magnetic beads (magnetic beads conjugated with β-conglutin) were added and the volume was brought to 100 µL with the selection buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM MgCl2, pH 7.4) and the solution incubated for 30 min at 21ºC. The solution containing DNA that did not bind with the magnetic beads was removed, and after 3 washes in 500 µL of binding buffer the DNA bound to the magnetic beads was eluted twice with 50 µL Milli-Q water by denaturation at 95ºC for 3 min. After the second round a negative selection step was incorporated where the selected DNA (5 µL) was added to 5 µL of ethanolamine-blocked magnetic beads with no protein attached, and, as before, the volume was brought to 100 µL with the selection buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM MgCl2, pH 7.4) for incubation at 21ºC for 30 min and the unbound DNA was then used as template for the next cycle of SELEX.

Amplification of selected sequences: 100 µL of the PCR mixture contained 10 µL template, 0.5 mM $MgCl_2$ 0,1 µM primers, 0.2 mM dNTPs, 5 U Tfi DNA polymerase, and buffer for Tfi DNA polymerase. After a 5 min incubation at 95°C, 18 cycles pilot PCR were carried out using (i) 95°C, 30 s for denaturation; (ii) 58ºC, 30 s for annealing; (iii) 72ºC, 30 s for elongation, and finally 5 minutes at 72ºC. Single stranded DNA (ssDNA) was generated following PCR by addition of 2.5 U/µL of T7 Gene 6 Exonuclease [43], and after 2 hours incubation at 37ºC the reaction was stopped by denaturation of the enzyme by heating at 80ºC for 10 min, followed by ethanol precipitation to obtain highly purified ssDNA.

Monitoring the evolution of SELEX using ELONA and SPR: β-conglutin (10 µg/mL) was immobilized on NUNC Maxisorp microtitre plates using 50 mM carbonate buffer pH 9.6 for 1 h at 37°C, followed by a 1-h blocking with PBS-Tween (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4, 0.05% v/v Tween 20). The plates were manually washed three times with PBS-Tween. Following these steps aliquots of the same concentration of ssDNA from each of the SELEX cycles in the selection buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM MgCl2, pH 7.4) were added to each well of the microtitre plate and incubated for 30 min at 21ºC. The plates were then manually washed three times with PBS-Tween. 50 µL of 5 nM biotinylated reporter probe (5'-att tcg aga ttg ctc gac tcg tg-3'; 5’-biotinylated) was added to each well and incubated for 1 h at 21ºC, again in the selection buffer. The plates were then manually washed three times with PBS-Tween and following the addition of Streptavidin-HRP (0.02 µg/mL) and TMB substrate the reaction was stopped after 20 min with 1 M H_2SO_4, and measured at 450 nm using a Spectramax 340PC384 plate reader (Figure 3.1.).

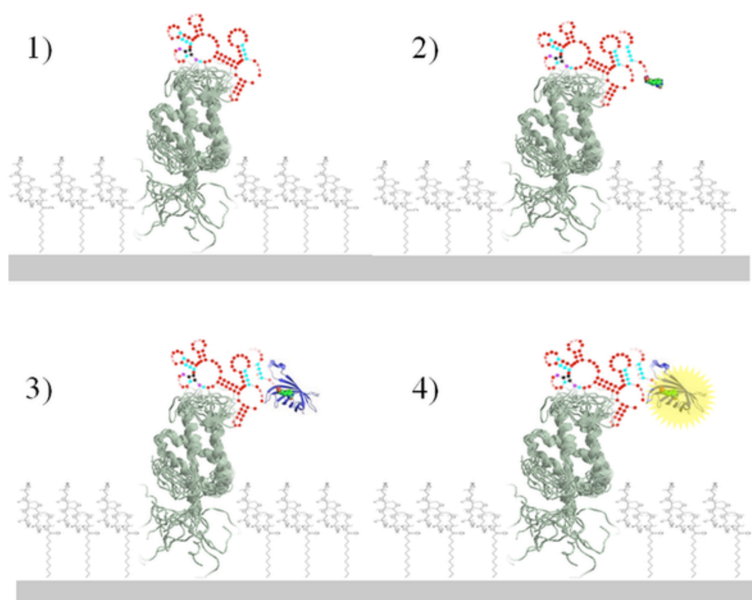

Figure 3.1. Schematic steps involved in the ELONA type assay. In step 1 the target protein is coated on the surface of a microtitre well and incubated with the aptamer pool. In step 2 biotinylated probe complementary to the constant 39 primer binding end of the 93-mer DNA is added and hybridises to any DNA that has bound to the immobilised target protein. In step 3, streptavidin labelled-horse radish peroxidase (HRP) is added and binds to the biotin label, and finally in step 4, tetramethylbenzidine substrate for the HRP is added and the resulting increase in absorbance is measured.

Cloning and Sequencing: DNA was cloned into the plasmid pCR2.1 using the TOPO TA Cloning® kit (Invitrogen, Spain) according to the manufacturer's instructions. Colonies were subsequently selected and grown overnight in a culture of 5 mL LB medium under vigorous shaking. Plasmid clones were purified with a QIAprep Spin Miniprep kit (Qiagen). Purified plasmid DNA were sequenced by the GenomeLab DTCS Quick Start Kit (Beckman Coulter) according to the manufacturer's instructions, and analyzed in a CEQ8000 Beckman Coulter instrument. The sequences derived were aligned using the Clustal software package of the GCG suite of molecular biology programs and CLC DNA workbench version 5.7.1.

Surface plasmon resonance (SPR) analysis: BIAcore 3000 (Biacore Inc.) with the Biaevalution software was used for the SPR experiments. Proteins of interest were immobilized, via amine coupling, on separate channels of a CM5 sensor chip. First the chip was activated by EDC/NHS followed by an injection of the protein (5 µL/min for 10 min). After immobilization of the protein any unreacted NHS esters were deactivated by

injecting an excess of ethanolamine hydrochloride followed by 75 mM NaOH to remove any non-specific adsorption. The DNA from each cycle of SELEX, as well the final aptamer candidates, were diluted in binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM MgCl2, pH 7.4) and injected for 6 min at a flow rate of 5 μL/min followed by 3 min stabilization and 10 min dissociation. The binding of DNA was analyzed through corresponding changes in the refractive index of optical signals, and expressed as resonance units (RU). All reagents and buffers were prepared in Milli-Q water and were previously filtered.

Secondary structure prediction: The secondary structure model of the sequences obtained was deduced using m-fold at 21ºC in 0.138 M [Na^{I}] and 1.5 mM [Mg^{II}] folding algorithm and QGRS Mapper, a web-based server for predicting G-quadruplexes in nucleotide sequences [44, 45].

Competitive ELONA assay: β-conglutin (10 μg/mL) was immobilized on NUNC Maxisorp microtitre plates in 50 mM carbonate buffer, pH 9.6, for 1 h at 37°C, followed by 1 h blocking with PBS-Tween (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4, 0.05% v/v Tween 20). The plates were then manually washed three times with PBS-Tween. In individual eppendorf tubes, serial dilutions of β-conglutin ranging from 5-100 μg/mL were incubated with Sequence 40 (100 nM) in the selection buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, pH 7.4) for 30 min at 21ºC and then added to the wells of the coated plate and incubated for 30 min at 21ºC. The plates were then manually washed three times with PBS-Tween. 50 μL of 5nM biotinylated probe (biotin-5'-att tcg aga ttg ctc gac tcg tg-3') was added to each well and incubated for 30 min at 21ºC in the selection buffer. The plates were again manually washed three times with PBS-Tween. Following the addition of Streptavidin-HRP (0.02 μg/mL) and after 30-minute incubation, TMB substrate was added, and 15 minutes later 1 M H2SO4 was added to stop the enzymatic reaction. Finally the absorbance was measured at 450 nm using a Spectramax 340PC[384] plate reader.

4. RESULTS AND DISCUSSION

Evaluation of β-conglutin conjugated magnetic beads

The attachment of the β-conglutin to magnetic beads was confirmed using peptide mass fingerprinting (PMF) using the positive controls Prostate Specific Antigen (PSA) and γ-conglutin (purified as described in [42]) with Q8IXI4 and Q9FSH9 accession number in UniProtKB and TreEMBL, respectively (Table 3.1.). Magnetic beads blocked with ethanolamine were used as a negative control. The results obtained from PMF demonstrated clear spectra with a high MOWSE score indicating immobilization of the pure target on the magnetic bead surface. Here the protein attached to the magnetic bead surface was directly digested with trypsin and the peptides produced were then analyzed using peptide mass fingerprinting, and the profile obtained in each spectra was then compared to the NCBI/UniProtKB/TrEMBL databases that contain the theoretical masses derived from the *in silico* tryptic digestion for millions of protein sequences. According to the number of peptide masses matched, including a minimum mass error tolerance of 50 ppm and using the MOWSE score algorithm, the peptide profile in the database were ranked, and the best score identified the target protein, confirming the coupling of the magnetic beads with the β-conglutin protein [42].

Table 3.1. MOWSE scores obtained in the Peptide mass fingerprinting of the protein-conjugated magnetic beads.

Protein name	MOWSE Score	Protein MW (Da)	pI	Accesion #	Species
β-conglutin (Lup an 1)	4.75E+13	62032	6.1	Q53HY0	*Lupinus albus*

In vitro selection: SELEX

The ssDNA library pool was heat-treated to denature any preformed structures and the SELEX procedure started with incubation of the library with the β-conglutin-conjugated magnetic beads in the binding buffer. Following the partitioning of bound from unbound DNA, the selected oligonucleotide pool was amplified using pilot PCR. In the pilot PCR a small aliquot of the SELEX pool was amplified in ranges of 5 to 20 PCR cycles in order to optimise the conditions required for amplification, maintaining the same amount of DNA for each cycle of SELEX, facilitating the use of the same number of molecules in each SELEX round. Once the PCR cycles required to maintain the starting amount of molecules for the next SELEX round was established the selected sequences were then amplified in the final PCR.

Following the amplification of the selected DNA from each SELEX cycle by PCR, double stranded DNA molecules were obtained. The T7 Gene 6 Exonuclease hydrolyzes duplex DNA non-processively in the 5'-3' direction from both 5'-phosphoryl or 5'-hydroxyl nucleotides by liberating oligonucleotides, as well as mononucleotides, until about 50% of the DNA is acid soluble. To generate single stranded DNA, the forward primer used in the PCR was modified with several phosphorothioates at its 5´end, which protected the forward primer terminated strand of the DNA duplex, liberating ssDNA for the next cycle of SELEX. Following the PCR of each SELEX cycle, the generated ssDNA was directly incubated with the β-conglutin conjugated magnetic beads as described for the first cycle.

After the second SELEX cycle, prior to the incubation with the protein-conjugated magnetic beads a negative selection step was included, where the amplified DNA, following ssDNA generation was initially incubated with negative magnetic beads (i.e. beads blocked with ethanolamine but without target). This negative selection removes the non-specific sequences that bind to the beads rather than to the target, increasing the stringency of the selection procedure. Pilot PCR was also carried out with oligonucleotides selected with the negative magnetic beads (without target) and with the positive magnetic beads protein-conjugated) for each SELEX cycle and was used to evaluate incremental affinity with each SELEX cycle. After 7 cycles of SELEX a much higher amount of DNA was obtained from the protein-conjugated beads as compared to the non-target conjugated beads, indicating that the DNA pool was becoming more selective towards the β-conglutin target.

Affinity and specificity studies

The evolution of the SELEX procedure was tested using enzyme linked oligonucleotide assay (ELONA) and surface plasmon resonance (SPR) using Biacore. For ELONA, in the first step the β-conglutin target was immobilised on NUNC Maxisorp microtitre plates and then incubated with the ssDNA generated after each cycle of SELEX (Figure 3.1.1.). For the second step a biotinylated probe with the same sequence as the reverse primer, which hybridises to the 3' end of the selected sequences was added (Figure 3.1.2.), followed by streptavidin-HRP, (Figure 3.1.3.). TMB substrate was then added, providing a colorimetric signal proportional to the amount of aptamer bound (Figure 3.1.4.). This experiment ran the risk that if the reverse primer was involved in the three-dimensional structure of the selected aptamer that no binding of the biotinylated probe would be observed. However, if binding is observed it can be assumed that the primer is not involved in target binding and in truncation studies, could most probably be removed without affecting the affinity of the aptamer. As can be seen in Figure 3.2., binding was in fact observed, and, in agreement with the PCR results, after the 7th cycle of SELEX, the selected DNA was observed to have increased affinity towards the β-conglutin target.

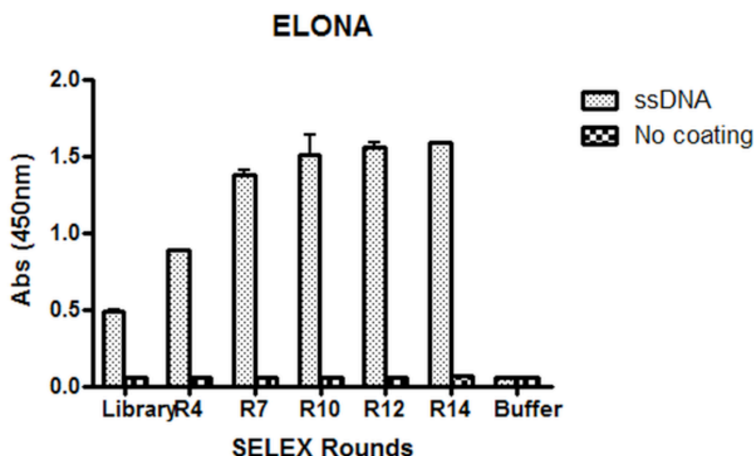

Figure 3.2. SELEX evolution by ELONA assay. Evaluation of evolution using ELONA monitoring of interaction between immobilised target and DNA isolated after rounds 4 (R4), 7 (R7), 10 (R10), 12 (R12) and 14 (R14), as compared to the interaction with the initial library. A control in the absence of coated target for each of the rounds (No coating) and a background control that refers to the response in the presence of buffer alone (Buffer).

The evolution of the DNA pool towards the target protein was also confirmed using surface plasmon resonance with a Biacore3000 and a CM5 chip where the β-conglutin was attached via amino coupling to the chip surface. In order to check the specificity of the selected DNA, gliadin crude extract, and ethanolamine (blocking agent) were used as negative controls. The selectivity and specificity of the evolving DNA from the SELEX cycles was clearly demonstrated as specific binding was only observed for the β-conglutin channel (Figure 3.3.).

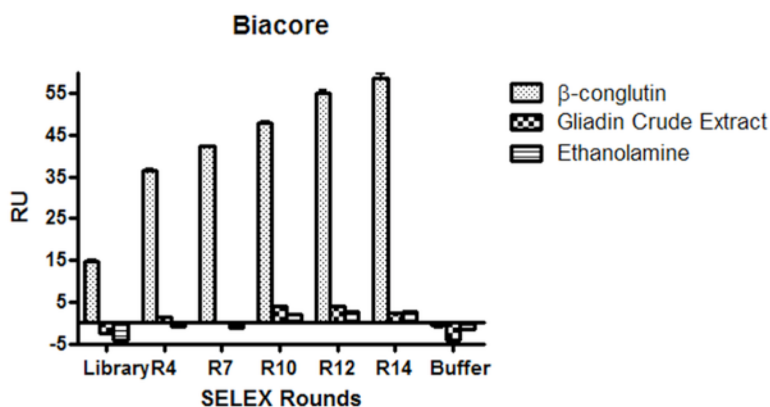

Figure 3.3. SELEX evolution by SPR. RU reached at the plateau of the association curve for all different channels of the Biacore CM5 chip with DNA isolated from various rounds of SELEX (4, 7, 10, 12 and 14) using channels coated with β-conglutin, gliadin crude extract and simply blocked with ethanolamine.

In agreement with the ELONA and PCR results, after the 7th SELEX cycle there is a definitive increase in the affinity of the selected DNA for the target reaching a plateau after the 12-13th cycle (Figure 3.3.). Once evolution had been established, cloning and sequencing was carried out to define the consensus motif.

Cloning and sequencing

The first step of the cloning was to amplify the SELEX round to be cloned in the same conditions described in the SELEX process. The presence of 3´A-overhangs on the PCR product was incorporated by including a 30 minute extension at 72°C after the last PCR-cycle. These ensure that all PCR products were full length and 3´-adenylated to facilitate ligation of the insert into the plasmid. The lag time extension after the last PCR-cycle favours the nontemplate-dependent terminal transferase activity of the *Tfi* DNA polymerase to add a single deoxyadenosine (A) to the 3´ ends of PCR products and a linearized vector with single, overhanging 3´ deoxythymidine (T) residues allows the PCR inserts to ligate efficiently with the plasmid vector. Following amplification electrophoresis was carried out and the band obtained in the agarose gel was excised and purified for insertion into the plasmid. To ensure that the plasmid contained the aptamer sequence, a PCR was carried out and if the clones contained the aptamer, a 93 bp product should be obtained and thus only clones with a ≈100 bp band were sequenced.

The alignment of the aptamer sequences led to the identification of two sequence families in 50 individual aptamer clones as can be seen in the phylogenetic tree, which describes the relationship between the two groups of sequences obtained (See Figure 3.S1. and 3.S2.). This indicates that the initial population of sequences had decreased

from 10^{14} different molecules to just a couple of sequence families, indicative of the convergence of potential aptamers in the resulting pool. Consensus motifs were identified from these sequence families and were purchased for analysis of their affinity towards the target protein.

SPR evaluation of candidate aptamers

The identified consensus motif sequences were evaluated using SPR. Of the 27 sequences listed in Figure 3.S1., which were evaluated for binding to the β-conglutin target, significant binding was observed for sequences numbered **2** and **40**, detailed below:

S2: 5'-agc tga cac agc agg ttg gtg ggg gtg gct cac atc atg gta gaa tga ctg aac agc gtt gat taa aag gca cga gtc gag caa tct cga aat-3' and

S40: 5'-agc tga cac agc agg ttg gtg ggg gtg gct tcc agt tgg gtt gac aat acg tag gga cac gaa gtc caa cca cga gtc gag caa tct cga aat-3'.

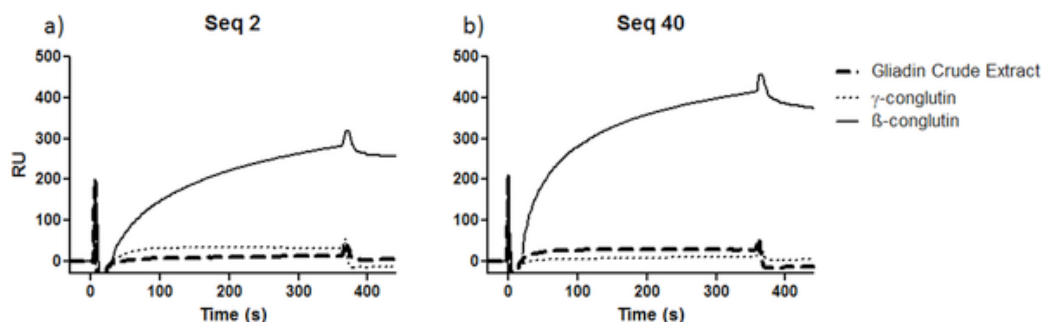

Figure 3.4. SPR Sensorgrams of the aptamer sequences obtained. a) SPR Sensorgram obtained for Sequence 2 at 10 mM passed through the CM5 Biacore chip surface showing the interaction with the immobilized target (Channel 1: β-conglutin) and negative controls (Channel 2: γ-conglutin, Channel 3: Gliadin Crude Extract); b) SPR Sensorgram obtained for Sequence 40 at 10 mM passed through the CM5 Biacore chip surface showing the interaction with the immobilized target (Channel 1: β-conglutin) and negative controls (Channel 2: γ-conglutin, Channel 3: Gliadin Crude Extract).

The signal observed in the negative control channels with immobilised gliadin and γ-conglutin, was negligible for both sequences, demonstrating the high specificity of these aptamer candidates (Figure 3.4. a) and b)). The K_D of each of Sequence **2** and Sequence **40** was obtained by analyzing the binding of a range of concentrations (100 nM to 10 μM) with β-conglutin, using a one to one Langmuir binding model, without mass transfer effect. The resulting K_D were 5.15 x 10^{-7} M, and 3.6 x 10^{-7} M for sequence **2** and **40**, respectively and a good fit to the model was obtained as

demonstrated by the χ^2 values of 0.0386 and 0.0681 obtained for each sequence (Table 3.2.). [**Note**: The Chi^2 value is a standard statistical measure of the closeness of fit of data to the model used for elucidation of the K_D, where for good fitting to ideal data, χ^2 is of the same order of magnitude as the noise in RU, typically <2].

Table 3.2. Dissociation constants obtained in Biacore with relevant statistics of the aptamers obtained.

Aptamer	Sequence	Model	K_D (nM)	X^2
2	5'-agc tga cac agc agg ttg gtg ggg gtg gct cac atc atg gta gaa tga ctg aac agc gtt gat taa aag gca cga gtc gag caa tct cga aat-3'	Langmuir Binding	515	0.0386
40	5'-agc tga cac agc agg ttg gtg ggg gtg gct tcc agt tgg gtt gac aat acg tag gga cac gaa gtc caa cca cga gtc gag caa tct cga aat-3'	Langmuir Binding	360	0.0681

The secondary structures of sequence **2** and **40** were modelled using m-fold software [44] and it was predicted that both sequences contain significant secondary structure, including protruding loops and stems (Figure 3.S3.). Furthermore, a QGRS-mapper was used to predict putative G-quadruplexes formed from G-Rich Sequences in sequence **2** and **40** (Figure 3.S4.), revealing a high probability of the presence of G-quadruplex structures in both sequences. Detailed ongoing studies involving NMR and circular dichroism analysis will provide a more exact description of the aptamer structure, but the QGRS-mapper clearly indicates that G-quartets are involved in the aptamer structure, a property that can be exploited when engineering a molecular beacon structure.

Competitive ELONA assay

In order to demonstrate the functionality of the selected aptamer, a competitive ELONA assay was developed for the quantitative detection of β-conglutin. This assay was based on the use of β-conglutin immobilised on a microtitre plate, which then "competed" with β-conglutin analyte for binding to the selected aptamer (sequence 40), where, similiar to competitive ELISA formats, in the presence of higher concentrations of the β-conglutin analyte, less aptamer bound to the immobilised β-conglutin, resulting in a lower signal. A calibration curve between 0 and 4.8 μM of β-conglutin (Figure 3.5.) was obtained, with an EC50 value of 392.8 nM. This EC50, which is the half concentration of a ligand where the response (or binding) is maximal, is in agreement with the K_D values obtained using surface plasmon resonance (Table 3.2.). The LOD obtained was 153 nM, and the r^2 was 0.999, clearly demonstrating the functionality of the selected aptamer for the quantitative detection of β-conglutin, the identified Lup an 1 allergen.

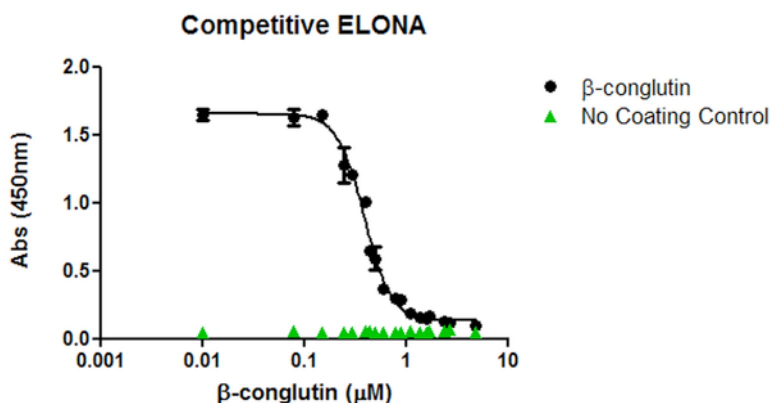

Figure 3.5. ELONA assay. Detection of β-conglutin by ELONA using Sequence 40 in SELEX Binding Buffer at 100 nM final concentration.

In conclusion, we report on the use of a SELEX procedure based on the use of protein-conjugated magnetic beads for the generation of aptamers able to bind specifically to the lupin β -conglutin allergen (Lup an 1). The final aptamers obtained detect the allergen Lup an 1 with high affinity and specificity capable of distinguishing it from other possible proteins present in flour, e.g. gliadin, or other globulin proteins present in lupin such as γ -conglutin. Furthermore the secondary structures of both sequences were predicted and evaluated using the GQRS-Mapper obtaining high G-score value for both sequences, indicative of the presence of G-quadruplexes. Finally the applicability of the aptamers obtained had been demonstrated by approaching a competitive ELONA assay. Further work will involve the elucidation of the exact structure of the aptamer and its truncated forms and other applications of those aptamers for the analysis of the Lup an 1 allergen, β-conglutin, in foodstuffs.

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6. SUPPORTING INFORMATION

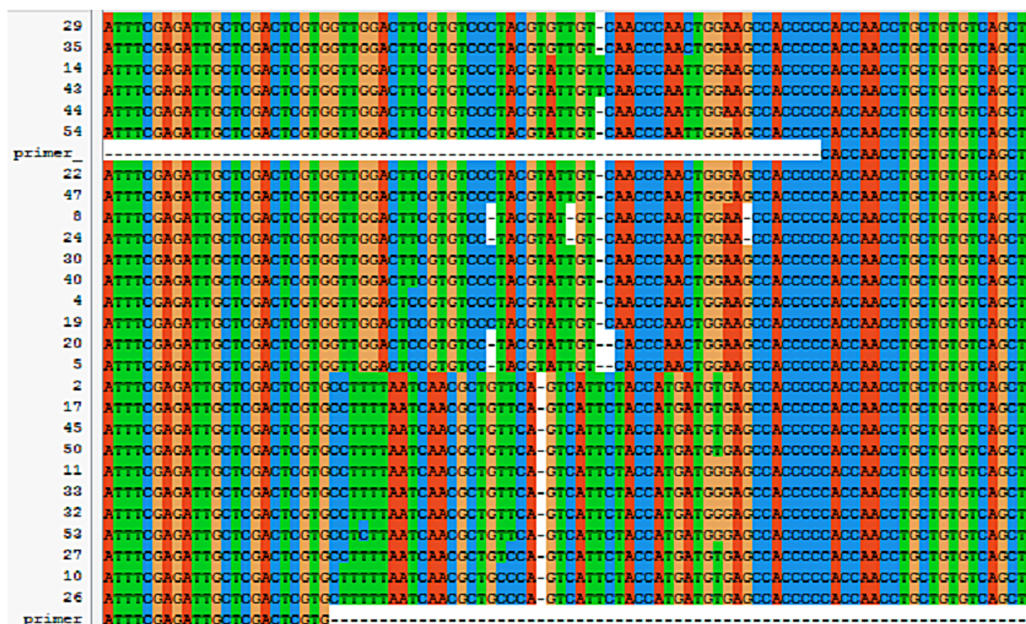

Figure 3.S1. Alignment of cloned sequences in clustalW.

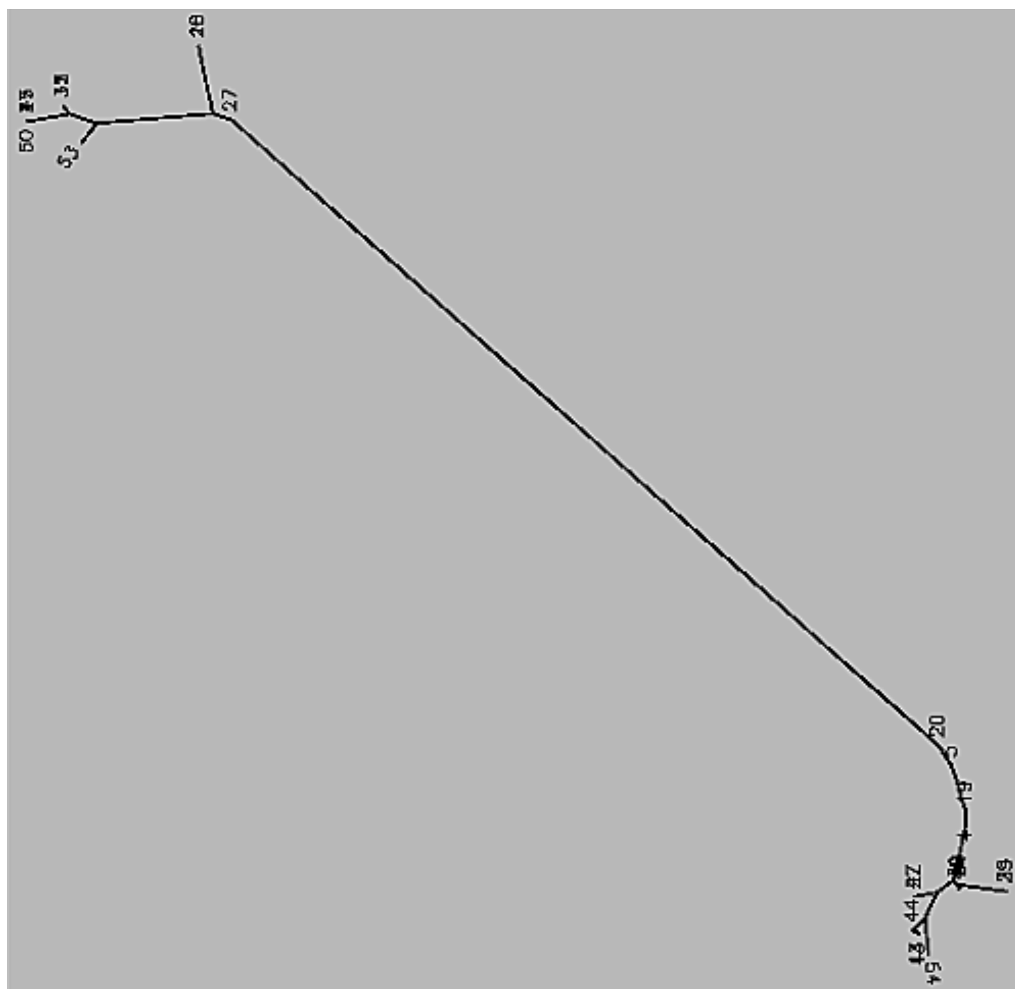

Figure 3.S2. Filogenetic tree showing the relationship of the two groups of sequences, sequenced in the cloning step.

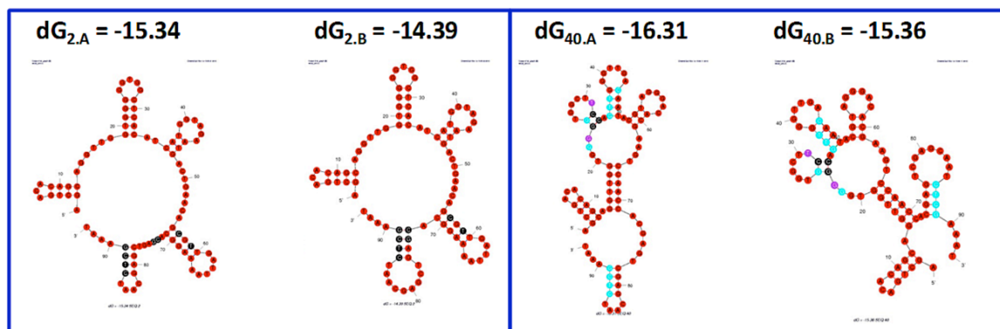

Figure 3.S3. Secondary structure prediction using m-fold software. Sequence 2 on the left, and Sequence 40 on the right.

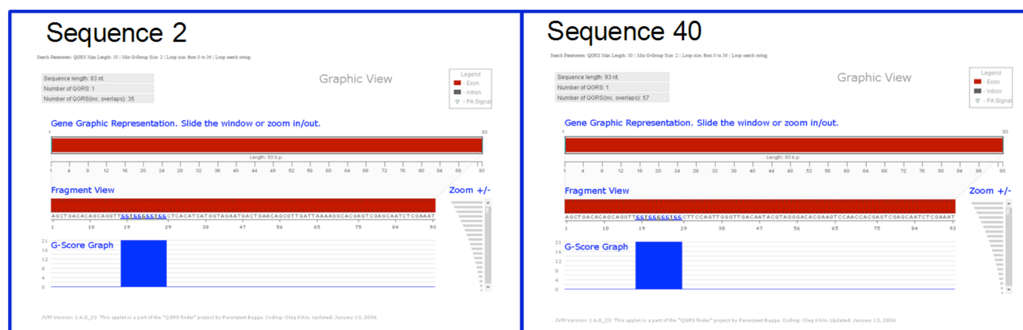

Figure 3.S4. Prediction of Guanine Tetrades. G-Score Graph for G-Quadruplex structure prediction using QGRS-mapper software, which indicates the probability of finding a G-rich motif capable of forming a G-quadruplex structure. Sequence 2 is shown on the left and Sequence 40 on the right.

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Dipòsit Legal: T. 1067-2012

CHAPTER 4

APTA-PCR FOR ULTRASENSITIVE DETECTION OF β-CONGLUTIN

UNIVERSITAT ROVIRA I VIRGILI
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1. ABSTRACT

Recently β -conglutin has been described as allergen Lup an 1 by the IUIS (International Union of Immunological Societies) and lupin foodstuff requires mandatory advisory labelling on foods sold in the European Union, in reaction to the increasing number of severe cases of lupin allergies reported during the last decade. In this paper we report the ultrasensitive detection of the anaphylactic allergen β -conglutin (Lup an 1) using Apta-PCR, exploiting competition between surface immobilized β -conglutin and solution-based target analyte for binding to the S40 β-conglutin aptamer in a competitive assay. Subsequently, by real time PCR analysis the amount of β-conglutin is calculated amplifying the bound aptamer, with a detection limit of 6 pM (r^2 0.999) and cross-reactivity against γ-conglutin or gliadin was observed to be negligible.

2. INTRODUCTION

Lupin is an herbaceous plant of the leguminous family belonging to the genus *Lupinus*. Lupin has been used as human food and animal feed since ancient times. Currently lupin flour and seeds, which are widely available as snacks are also used in bread, cookies, pastry, pasta, sauces, as well as in beverages as a substitute for milk or soy [1]. Lupin flour is a good emulsifying, rich protein source, and has many advantageous nutritional properties being a good controller of cholesterolemia as well as glucose haematic levels [2], and the use of γ-conglutin in tablets for the treatment of diabetic patients has recently been patented [3].

However there have been a response to the increasing number of severe cases of lupin allergies reported during the last decade [4-7], and as result lupin was recently added to the list of substances requiring mandatory advisory labelling on foodstuffs sold in the European Union. According to the Commission Directive 2006/142/EC Annex IIIA of Directive 2000/13/EC [8], all products containing even trace amounts of lupin must be labelled correctly. Furthermore β-conglutin has recently been designated as the Lup an 1 allergen by the International Union of Immunological Societies (IUIS) allergen nomenclature subcommittee [9].

Aptamers are artificial nucleic acids ligands that have been generated against a wide range of diverse targets. Aptamers are selected from a large population of random sequences in a synthetically produced library of nucleic acids by iterative rounds of systematic binding, competition, selection, amplification, and enrichment in a process termed SELEX, the Systematic Evolution of Ligands by Exponential Enrichment [10]. Since the first descriptions of *in vitro* selection technique for the isolation of specific nucleic acid sequences was reported by three laboratories [10-12], there have been a plethora of reports describing aptamers and their applications including their use in analysis where aptamers have been exploited as biomolecular recognition elements in a wide variety of applications, ranging from food quality control to clinical diagnostics [13]. Aptamers possess unique characteristics as compared to antibodies due to their nucleic acid nature, highlighting their superiority in terms of flexibility. Examples of this include their use in displacement assays [14], molecular beacons [15], and apta-PCR [16].

There have been several reports of aptamers for food safety, including the detection of biotoxins such as the mycotoxins fumonisin B [17], endotoxin [18], and several antibiotics with detection limits ranging from nanomolar to micromolar, e.g. Kanamycin A [19] and B [20], neomycin [21], tetracycline [22], chloramphenicol [23]. Furthermore aptamers have been used for the detection of various bacterial pathogens, including *Salmonella typhimurium* [24], *Escherichia coli O157:H7* [25] *Listeria monocytogenes* [26] and *Staphylococcus aureus* [27]. For the detection of food allergens, Lysozyme was the first example of a food allergen were RNA and DNA aptamers were selected

[28, 29], with equilibrium dissociation constants of 2.8 ± 0.3 nM and 0.8 ± 2.0 nM respectively, measured by fluorescence anisotropy at 25ºC [30].

Aptamers have been applied in a wide range of affinity amplification assays exploiting the predisposition of nucleic acid aptamers to an assay format that combines the selectivity of the aptamers with the efficiency of nucleic acid amplification techniques, producing an impressive signal enhancement with considerably lower detection limits.

The first report integrating the sensitivity of nucleic acid amplification with an immunoassay, in a technique known as Immuno-Polymerase Chain Reaction (Immuno-PCR), was in 1992 [31]. The use of a reporting antibody labelled with DNA, either directly, or via a biotin-streptavidin bridge, improved the sensitivity of a conventional immunoassay and enhanced the detection limits by up to 100 000-fold [32-35]. However the technique suffer some important drawbacks, such as difficulties in labelling the antibody with nucleic acids and, furthermore, this linkage, either directly to antibodies or via biotin-streptavidin linker bridges is prone to a lack of precision often resulting in uneven numbers of oligos per antibody, resulting in high rates of error and affecting sensitivity [36, 37].

Additionally, following the immunorecognition step, the DNA needs to be separated from the antibody for subsequent amplification. Aptamers do not require to be conjugated to a label as they can inherently act both as detecting and reporting molecule, simply by flanking the aptamer with two primer sequences, thus avoiding the problems with immuno-PCR. In aptamer affinity amplification methods, the antibody labelled with an oligonucleotide used in immuno-PCR is replaced by an aptamer ready to be amplified. Despite the fact that the technique has only been reported recently for the first time, there are already several different formats described in literature, where different sandwich formats and a variety of amplification methods have been reported.

Fisher *et al.* demonstrated detection of thrombin via Rolling Cycle Amplification (RCA) and real time quantitative PCR (qPCR) [38]. By means of different concentrations of thrombin modified magnetic micro-particles to bind the TBA flanked by two primer regions for amplification, the group achieved a 2nM detection limit using the Rolling Cycle Amplification (RCA) and as low as a few hundred fM using qPCR. Lee and co-workers described an antibody/aptamer mixed sandwich to detect up to 10 *E.coli* cells/mL *via* qPCR [39]. In an interesting report, Waga's team achieved the selection of an RNA aptamer against the constant region (Fc) of rabbit IgG [40] to be used as a reporter molecule that could be taken advantage of in a multitude of assays where rabbit IgG antibodies were used. Detection was achieved via qPCR [41], highlighting the immense potential of the simple combination of an aptamer with antibody in affinity amplification assays.

The approach reported by Pinto et al. [33] exploits an immobilized thrombin-binding aptamer (TBA29), used as capture aptamer, on a streptavidin- coated microtiter plate. Consequently the captured thrombin molecules were incubated with a second thrombin-binding aptamer (TBA15). The reporter aptamer (TBA15) was modified by flanking regions to include PCR primers for, subsequent amplification using Real Time PCR. This reported assay allowed the simultaneous recognition and quantification of thrombin molecules captured on solid surfaces, demonstrating a massive improvement (20000-fold enhancement) in terms of detection limit, when compared with methods previously described [16]. This approach is applicable to all aptamers, independent of whether there multiple aptamers exist against the target, and the technique can be applied to the detection of very low concentrations of target analytes, which is garnering ever-increasing importance in pre-emptive medicine and the early diagnosis of disease [16].

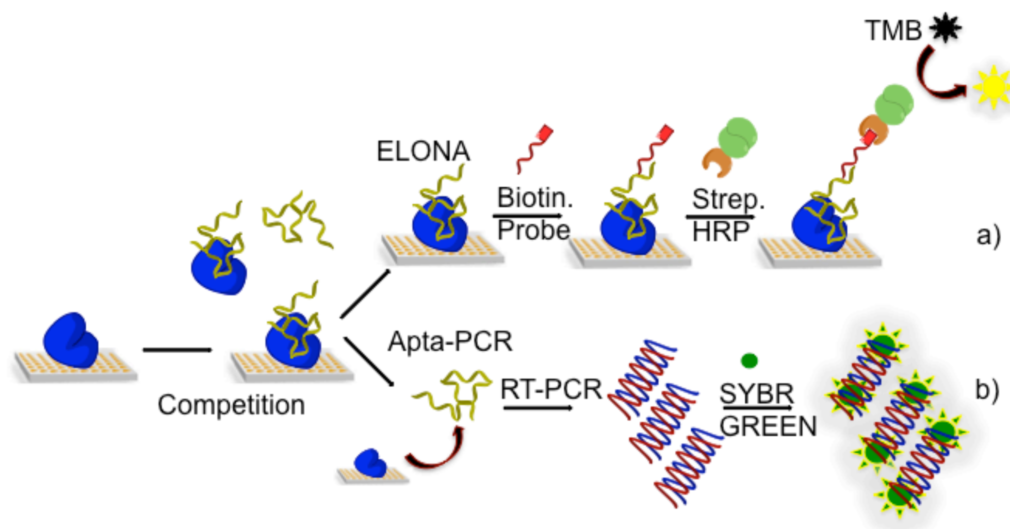

Figure 4.1. Schematic steps approached for both ELONA and Competitive Apta-PCR.

In this work, the ultrasensitive detection of β-conglutin is reported by exploiting the new method of Real Time Apta-PCR as described by Pinto et al. [16]. As can be seen in Figure 4.1, following competition between β-conglutin in solution and β-conglutin on plate surface, aptamers can be used for detection via ELONA (a) or via Apta-PCR (b). In ELONA a biotinylated probe complementary to the aptamer that hybridizes to an aptamer region not involved in target recognition was used. Streptavidin labelled with HRP was then added, following addition of TMB substrate and colour evolution was monitored. On the other hand, via Apta-PCR, aptamers are eluted from the plate surface following competition and are amplified by RT-PCR. SYBR Green then binds to the dsDNA generated during the amplification producing a measurable fluorescent

signal. In this work the S40 β-conglutin aptamer applied for the ultrasensitive detection of the lupin anaphylactic allergen.

Table 4.1. Lupin aptamer and primers sequences (from 5' to 3') [42].

		Sequence
β-conglutin Aptamer	**S40**	agc tga cac agc agg ttg gtg ggg gtg gct tcc agt tgg gtt gac aat acg tag gga cac gaa gtc caa cca cga gtc gag caa tct cga aat
Forward primer	**SLF**	agc tga cac agc agg ttg gtg
Reverse primer	**SLR**	att tcg aga ttg ctc gac tcg tg'

3. MATERIALS AND METHODS

Aptamer and primer sequences. Oligonucleotides (HPLC purified and provided lyophilized) were synthesized by Ella Biotech GmbH (Martinsried, Germany). Oligonucleotides and reagents were used as purchased. All solutions were prepared in high purity water obtained from a Milli-Q RG system (Barcelona, Spain). Details of the sequences of the S40 β-conglutin aptamer and the reverse and forward primers are detailed in Table 4.1.

Surface plasmon resonance (SPR) analysis. BIAcore 3000 (Biacore Inc.) with the Biaevalution software was used for the SPR experiments. Proteins of interest (i.e. β-conglutin and potential interferents γ -conglutin and gliadin) were immobilized, via amine coupling, on separate channels of a CM5 sensor chip. First the chip was activated by EDC/NHS followed by injection of the protein (5 µL/min for 10 min). After immobilization of the protein, any unreacted NHS esters were deactivated by injection of an excess of ethanolamine hydrochloride, followed by 75 mM NaOH to remove any non-specific adsorption. The S40 β-conglutin aptamer diluted in binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM MgCl2, pH 7.4) was injected for 6 min at a flow rate of 5 µL/min followed by 3 min stabilization and 10 min dissociation. The binding of DNA was analyzed through corresponding changes in the refractive index of optical signals, and expressed as resonance units (RU). All reagents and buffers were prepared in Milli-Q water and were previously filtered [42].

Materials and instrumentation. Oligonucleotides were synthesized by ELLA-Biotech. Salmon Sperm DNA and Phosphate buffered saline–Tween 20 (PBS-T) were purchased from Sigma. 96 well microtitre plates were obtained from Thermo Scientific (Fisher Scientific). Real Time PCR analyses were performed on an Applied Biosystems HT-7900 using Power SYBR Green kit (Applied Biosystems).

Competitive ELONA assay. β-conglutin (10 µ g/mL) was immobilized on NUNC Maxisorp microtitre plates in 50 mM carbonate buffer, pH 9.6, for 1 h at 37°C, followed by 1 h blocking with PBS-Tween (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4, 0.05% v/v Tween 20). The plates were then manually washed three times with PBS-Tween. In individual eppendorf tubes, serial dilutions of β-conglutin ranging from 0 to 20 µM were incubated with the S40 β-conglutin aptamer (100 nM) in 10 mM

phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, pH 7.4 for 30 min at 22°C and then added to the wells of the coated microtitre plate and incubated for 30 min at 22°C. The plates were then manually washed three times with PBS-Tween. 50 µL of 5 nM biotinylated probe (biotin-5′-att tcg aga ttg ctc gac tcg tg-3′) was added to each well and incubated for 30 min at 22°C. The plates were again manually washed three times with PBS-Tween, and subsequently Streptavidin-HRP was added (0.02 µg/mL) and after 30 minutes incubation, TMB substrate was added, and 15 minutes later 1 M H_2SO_4 was added to stop the enzymatic reaction. Finally the absorbance was measured at 450 nm using a Spectramax 340PC[384] plate reader. Gliadin was used as a negative control.

Apta-PCR assay. PBS-Tween (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4, 0.05% v/v Tween 20) buffer was used for all binding and washing steps of the assay, and all incubations were carried out at 37ºC blocking under shaking conditions. 140 nM of β-conglutin was added to each well of a Nunc microtiter plate for 30 min. The plate was then washed three times and blocked for 30 min at 37ºC. Separately, a range of concentrations of β -conglutin from 0 to 20.0 µ M were incubated with the S40 β-conglutin aptamer (1 nM) for 30 min at 37ºC. The aliquots were then added to the coated plate and again incubated at 37ºC for 30 min. Following thorough washing the bound reporter aptamer was then eluted by heating to 95ºC for 5 min and the eluate was directly used as template in Real Time PCR.

Real Time PCR analysis. Real Time PCR reactions were performed in 20 µL volume in an Applied Biosystems 7900-HT Fast Real Time PCR system with Power SYBR Green kit (Applied Biosystems). The results were analyzed by Applied Biosystem SDS 2.4 Analysis software to calculate the amount of aptamer present in the sample. The PCR cycle consisted of an initial denaturation step at 95ºC for 2 min followed by 40 cycles of 95ºC for 30 s, 58ºC for 30 s and 72ºC for 30 s.

4. RESULTS AND DISCUSSION

In the competitive ELONA, β-conglutin is immobilized and competes with β-conglutin target analyte in solution β-conglutin for binding to the S40 β-conglutin aptamer which must be added in a constant concentration. Similar to competitive ELISA formats, in the presence of higher concentrations of the β-conglutin analyte, less aptamer binds to the immobilized β-conglutin, resulting in a lower signal [42]. The specificity and the dissociation kinetic constant (K_D $3.6{\times}10^{-7}$ M) of the S40 β-conglutin aptamer had previously been evaluated using Surface Plasmon Resonance (SPR) [42], and the calibration curve, had a dynamic range from 0 to 4.8 μM of β-conglutin with an EC50 value of 392.8 nM, and a LOD of 153 nM (r^2 was 0.999) (Fig. 4.2.). The cross-reactivity of the aptamer was negligible using gliadin as negative control.

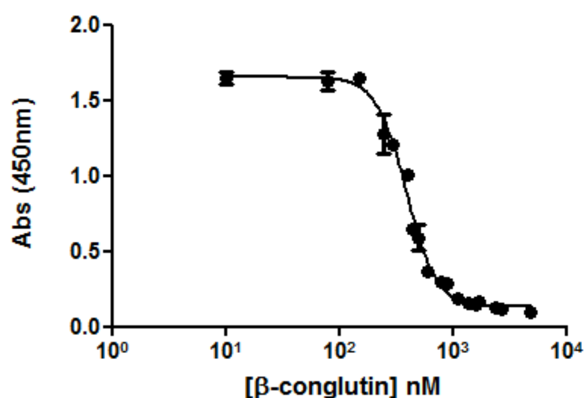

Figure 4.2. Detection of β-conglutin by ELONA using S40 β-conglutin aptamer in PBS-Tween (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4, 0.05% v/v Tween 20) at 100 nM final concentration. The errors bars represent the Standard Deviation of 3 repetitions.

In order to improve the detection limit, the inherent nucleic acid nature of the S40 β-conglutin aptamer was exploited as a combined biomolecular interaction and reporter element. Furthermore to elucidate the optimum conditions for the competitive Apta-PCR, the concentrations of coating β-conglutin and the S40 β-conglutin aptamer were varied in a type of checkerboard titration and a coating of 140 nM gave maximum binding of the S40 β-conglutin aptamer becoming saturated at 10 nM. A concentration of 1 nM S40 β-conglutin aptamer was thus chosen to be optimal as a limiting concentration of S40 β-conglutin aptamer is required to facilitate an effective competitive assay (Fig. 4.3.).

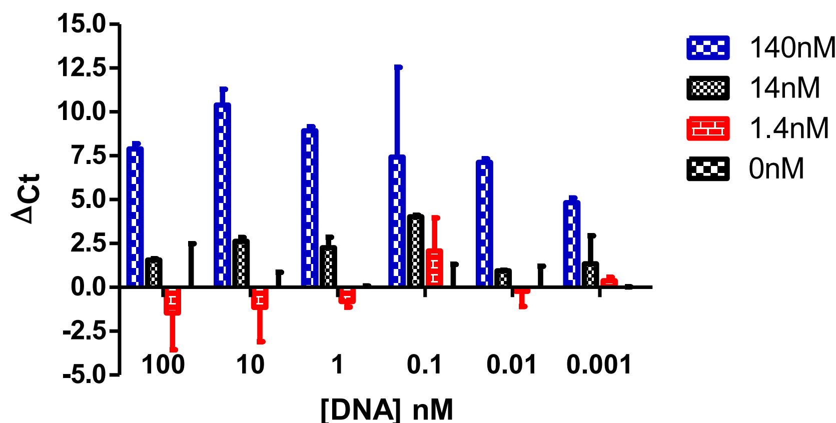

Figure 4.3. Apta-PCR optimization The errors bars represent the Standard Deviation of 3 repetitions.

Following optimization the calibration curve was prepared. Exploiting competition between surface immobilized β-conglutin and solution-based target analyte for binding to the S40 β-conglutin aptamer, in a similar manner to the competitive ELONA, with the exception that following competition the bound aptamer was heat eluted and used as a template in RT-PCR, where at higher concentrations of target β-conglutin, lower levels of the S40 β-conglutin aptamer would be available for binding to the surface. The ΔCt is the difference between the Cycle thresholds of the sample and the blank sample and the blank is the sample containing 1 nM S40 β-conglutin aptamer not exposed to the target. As can be seen in Figure 4.4, by plotting the resulting ΔCt versus the concentration of β-conglutin the calibration curve obtained, had a dynamic range from 0 to 14.0 μM of β-conglutin with an EC50 value of 347 nM, and a LOD of 6 pM (r^2 was 0.9955).

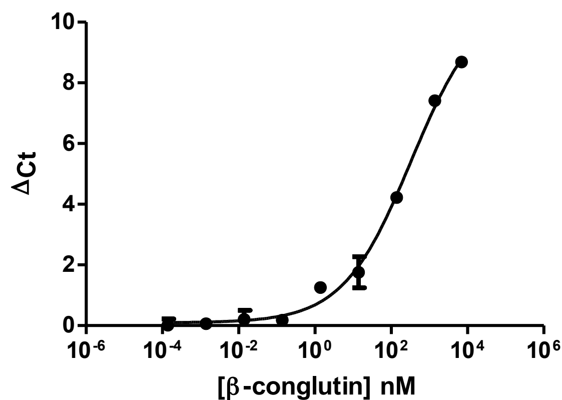

Figure 4.4. Calibration curve obtained for β-conglutin by Apta-PCR competitive assay. The errors bars represent the Standard Deviation of 3 repetitions.

A cross-reactivity study was carried out using 140 nM of γ-conglutin and gliadin, which were selected as the former is another subunit found in lupin and the later is chosen as lupin flour is used in gluten free products. As can be seen in Figure 4.6, there is negligible cross-reactivity, highlighting the specificity of the S40 β-conglutin aptamer.

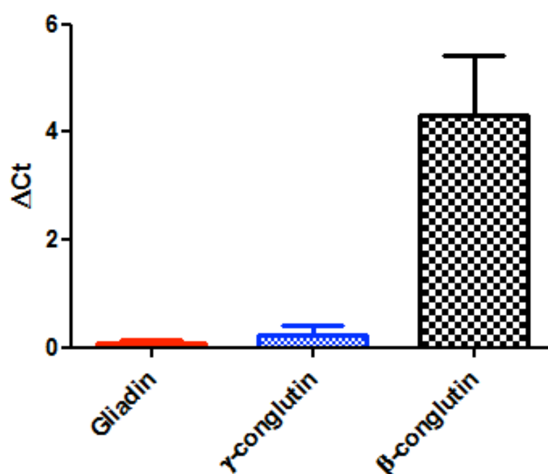

Figure 4.5. Cross-reactivity assay for the detection of β-conglutin at 140 nM using Apta-PCR competitive assay. Gliadin crude extract and γ-conglutin were used as negative controls, at 140 nM.

5. CONCLUSION

The first Real Time Apta-PCR using the S40 β-conglutin aptamer is reported. In this paper we report the ultrasensitivite detection of the anaphylactic allergen β-conglutin (Lup an 1) using Apta-PCR, exploiting competition between surface immobilized β-conglutin and solution-based target analyte for binding to the S40 β-conglutin aptamer in a competitive assay. Subsequently, by real time PCR analysis the amount of β-conglutin is calculated amplifying the remaining aptamer on the surface. Achieving a low detection limit of 6 pM (r^2 0.9955) with negligible cross-reactivity against γ-conglutin or gliadin. The case of β-conglutin the detection limit achieved vastly enhanced as compared to ELONA, decreasing from 153 nM to 6 pM, representing a greater than 25000-fold improvement.

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CHAPTER 5

TRUNCATION AND OPTIMIZATION OF SELECTED APTAMER FOR β-CONGLUTIN

UNIVERSITAT ROVIRA I VIRGILI
SELECTION, CHARACTERISATION AND ANALYTICAL APPLICATION OF DNA APTAMER AGAINST
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Dipòsit Legal: T. 1067-2012

1. ABSTRACT

β-conglutin is an important allergen found in lupin flour and seeds, which are widely available as snacks. Lupin flour is also used in bread, cookies, pastry, pasta, sauces, as well as in beverages as a substitute for milk or soy. In this paper we report the characterization and structural optimization of aptamers that specifically bind to β-conglutin. Through comparing the predicted secondary structures of the aptamers, a hairpin structure with G-rich loop was determined to be the binding motif of these aptamers. The highest affinity was observed with a truncation resulting in an 11-mer sequence that had an apparent equilibrium dissociation constant (Kd) of 5.88×10^{-10} M. This 11-mer sequence was demonstrated to have high specificity for β-conglutin and showed no cross-reactivity to closely related proteins, including γ-conglutin or gliadin. Further work will focus on the elucidation of the structure of this truncated highly specific and sensitive truncated aptamer for the Lup an 1 allergen β-conglutin.

2. INTRODUCTION

Lupin is an herbaceous plant of the leguminous family belonging to the genus *Lupinus*. Lupin flour and seeds, which are widely available as snacks, are also used in bread, cookies, pastry, pasta, sauces, as well as in beverages as a substitute for milk or soy. However, in response to the increasing number of severe cases of lupin allergies reported during the last decade, in December 2008 lupin was added to the list of substances requiring mandatory advisory labelling on foodstuffs sold in the European Union [1, 2]. Thus, all products containing even trace amounts of lupin must be labelled correctly [3] and the International Union of Immunological Societies (IUIS) allergen nomenclature subcommittee recently designated β-conglutin as the Lup an 1 allergen [4]. Currently available commercial enzyme linked immunosorbent assays exploit polyclonal antibodies that are not specific to β-conglutin [5] and reports in the literature only detail monoclonal IgG antibodies against α-conglutin and IgM antibodies against β-conglutin [6, 7], and there are no reports or commercial ELISAs for the specific detection of β-conglutin. There is thus a need for an analytical tool/method that can specifically detect the Lup an 1 allergen, β-conglutin [8].

Aptamers have been developed through the use of Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [9]. Aptamers are synthetic oligonucleotides that specifically bind with high affinity a wide range targets, from small molecules to whole cells [10-13]. DNA or RNA aptamers have gained attention as the next generation antibody-like molecules as research tools in molecular recognition for medical or diagnostic use. A full-length aptamer usually has three functional regions; Firstly the region that plays the role of contacting the target is normally approximately 10–15 nucleotides long, and has secondary structures such as hairpin loops, G-quartet loops, bulges, or pseudoknots. A second region contains nucleotides that do not directly contact the target but play an important role in supporting the interactions between the contacting nucleotides and the target. Whilst the third region comprises the nucleotides that do not bind to the target nor support the binding of the contacting nucleotides to the target [14], and they are regarded as nonessential nucleotides. It is always desirable to truncate the aptamer to eliminate the nonessential nucleotides after the upstream aptamer-selection process (i.e., downstream truncation). There are several reasons for carrying out downstream truncation. First, in some cases, elimination of nonessential nucleotides can increase the binding functionality of the aptamer presumably due to reduced steric hindrance [15]. Second, although the chemical synthesis of an oligonucleotide is a well-established technique, the synthesis of aptamers longer than 60 nucleotides is always more expensive and difficult to perform, and the shorter the aptamer, the more cost-effective it is. It is thus highly important to understand how selected aptamers interact with their targets, in order to elucidate the redundant motifs in the aptamer sequence.

However truncation may result in the removal of the non-binding domain and may actually interfere with the interaction between the aptamer and target protein by formation of complex secondary structures, and could eventually prevent the binding domain from folding into the desired conformation for binding to the target. Therefore, identifying the high binding affinity domains in the post-screened aptamer is a key step for producing potent aptamers of higher affinity/specificity [16].

Nuclear Magnetic Resonance (NMR) spectroscopy has been successfully applied to solve the structures of a range of aptamer or ligand-aptamer complexes. Using NMR spectroscopy techniques, the structure and dynamics of aptamers can be predicted [17, 18], and moreover, the characterization of aptamer conformational transitions and the structural changes due to the addition of cofactors, such as ions, small molecules or even proteins, could give an insight into the biomolecular reaction mechanisms [19, 20].

In the work reported here a combination of approaches were used in order to optimise the size of the aptamers selected against the lupin potential allergen Lup an 1, β-conglutin [21], which we refer to, as the S2 and S40 β-conglutin binding aptamers. Firstly primers were removed and the degree of interference with the interaction between the aptamer and its' target was probed [22]. Furthermore, a second truncation approach was carried out based on the analysis of simulated structures and characterization of aptamer segments. The use of algorithms to theoretically predict the secondary structures of aptamers can assist in determining which segments of the selected aptamer are essential sequences for binding [23, 24]. This technique has for instance been successfully used for the truncation of anti-hPTK7 aptamers [25]. Current algorithms predict the structures of nucleic acids in the absence of target molecules whereas it is possible for some, if not all, aptamers to undergo conformational changes upon binding to the target molecules [26]. Finally, following prediction of the structure using m-fold and GQRS-mapper, truncated sequences were generated accordingly and binding studies were used for determination of the specificity and affinity of these truncated aptamers using Surface Plasmon Resonance (SPR) [21, 27-36], and the K_D of the final truncated 11-mer aptamer was determined.

3. MATERIALS AND METHODS

Secondary structure prediction

The secondary structure model of the sequences obtained was predicted using m-fold at 22ºC in 0.138 M [Na^{I}] and 1.5 mM [Mg^{II}] and the QGRS Mapper, for predicting G-quadruplexes in nucleotide sequences [23, 24]. .

Surface plasmon resonance (SPR) analysis

A BIAcore 3000 (Biacore Inc.) with Biaevalution software was used for the SPR experiments using either protein or aptamer candidates immobilized on the surface. Proteins of interest (i.e. β-conglutin and potential interferents γ-conglutin and gliadin) were immobilized, via amine coupling, on separate channels of a CM5 sensor chip. First the chip was activated by EDC/NHS followed by an injection of the protein (5 μL/min for 10 min). After immobilization of the protein, any unreacted NHS esters were deactivated by injecting an excess of ethanolamine hydrochloride, followed by 75 mM NaOH to remove any non-specific adsorption. The various truncated sequences under evaluation were diluted in 10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, pH 7.4 and injected for 6 min at a flow rate of 5 μL/min followed by 3 min stabilization and 10 min dissociation.

The binding of protein/DNA was analyzed through corresponding changes in the refractive index of optical signals, and expressed as resonance units (RU). All reagents and buffers were previously filtered and dissolved in milli-Q water. All SPR measurements were performed in triplicate.

4. RESULTS AND DISCUSSION

Secondary Structure prediction

The first step for the truncation of the S2 and S40 β-conglutin aptamer was to remove the primer sequences at either the 3'-extreme (2.3, 40.3), or the 5'-extreme (2.2, 40.2), or at both extremes (2.1, 40.1) (Table 5.1).

Table 5.1. Full length aptamer sequences, truncated species and controls (5'-3' direction).

Label	Sequence (from 5' to 3')	Nucleotides	MW ($g \cdot mol^{-1}$)
S2	agc tga cac agc agg ttg gtg ggg gtg gct cac atc atg gta gaa tga ctg aac agc gtt gat taa aag gca cga gtc gag caa tct cga aat	93	28952
S40	agc tga cac agc agg ttg gtg ggg gtg gct tcc agt tgg gtt gac aat acg tag gga cac gaa gtc caa cca cga gtc gag caa tct cga aat	93	28905
SGQ	g gtg ggg gtg g	11	3509
S2.1	ggg gtg gct cac atc atg gta gaa tga ctg aac agc gtt gat taa aag g	49	15275
S2.2	ggg gtg gct cac atc atg gta gaa tga ctg aac agc gtt gat taa aag gca cga gtc gag caa tct cga aat	72	22378
S2.3	agc tga cac agc agg ttg gtg ggg gtg gct cac atc atg gta gaa tga ctg aac agc gtt gat taa aag g	70	21848
S40.1	ggg gtg gct tcc agt tgg gtt gac aat acg tag gga cac gaa gtc caa c	49	15228
S40.2	ggg gtg gct tcc agt tgg gtt gac aat acg tag gga cac gaa gtc caa cca cga gtc gag caa tct cga aat	72	22331
S40.3	agc tga cac agc agg ttg gtg ggg gtg gct tcc agt tgg gtt gac aat acg tag gga cac gaa gtc caa c	70	21801
TBA	ggt tgg tgt ggt tgg	15	4727

5'-agc tga cac agc agg ttg gtg-(N)49-cac gag tcg agc aat ctc gaa at-3' , the full length sequences include two primer hybridization sites and the centre random sequence.

In the first instance, β-conglutin was immobilized on a CM5 chip and sequences corresponding to the truncated S2 and S40 aptamers detailed in Table 5.1 were passed over the chip surface. As can be clearly seen in Figure 5.1.a and 5.1.b, the sequences present at the 5'-extreme (S2.2 and S40.2) do not appear to participate in target binding, whilst sequences present in the 3'extreme are obviously essential for binding (S2.3 and S40.3).

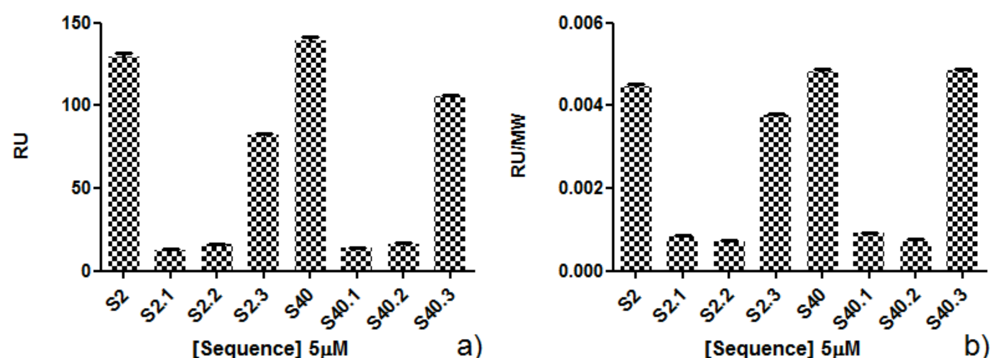

Figure 5.1. Resonance Units obtained at the end of the dissociation of the sensorgram for the truncated sequences. a) RU reached at the plateau of the association curve of each molecule on a Biacore CM5 chip with target (β-conglutin). b) RU reached at the plateau of the association curve normalized by the molecular weight of each molecule on a Biacore CM5 chip with target (β-conglutin).

The procedure used for the second truncation approach was based on the analysis of simulated structures. The use of algorithms to theoretically predict the secondary structures of aptamers can help to determine which segments of the aptamers are essential sequences for binding [23, 24] (Fig. 5.2-3). For instance, this technique has been successfully used for the truncation of anti-hPTK7 aptamers [25].

Firstly the S2 and S40 β-conglutin aptamers [21] were compared and 11 sequential nucleotides with 100% homology were found in both sequences and we refer to this 11-mer as the SGQ β-conglutin aptamer. To generate information on the composition and distribution of putative Quadruplex forming G-Rich Sequences in the SGQ β-conglutin aptamer, the QGRS-mapper was used (Fig. 5. S1-2). The G-quadruplex structure, also known as a G-quartet, is composed of stacked G-tetrads, which are square co-planar arrays of four guanine bases each. These interesting structures may be formed by repeated folding of a single nucleic acid molecule or by interaction of two or four strands and are generally very stable due to cyclic Hoogsteen hydrogen bonding between the four guanines within each tetrad [23]. The SGQ β-conglutin aptamer, was compared with the thrombin binding aptamer (TBA) [37-39] using the GQRS-mapper [23]. The G-Score is the statistical parameter used by the GQRS-

mapper software for predicting the probability of finding a G-quadruplex structure motif, the higher the G-Score the higher the probability is. The TBA, which has a well known and studied G-quadruplex structure achieves a GQ-score of 20 and the same range was obtained for the 11-mer truncated aptamer (G-Score 21). This preliminary analysis thus suggested the high probability of the presence of G-quadruplex structures in the motif.

The identification of this 11-mer G-quadruplex can also explain the results obtained in Figure 5.1. For the 2.1, 40.1, 2.2 and 40.2 truncated sequences, only seven bases of the identified 11-mer are present, with GGTG of the 11-mer having been truncated, thus preventing the G-quadruplex from forming, and consequently hindering target binding, whilst in the 2.3 and 40.3 sequences, the 11-mer is kept intact.

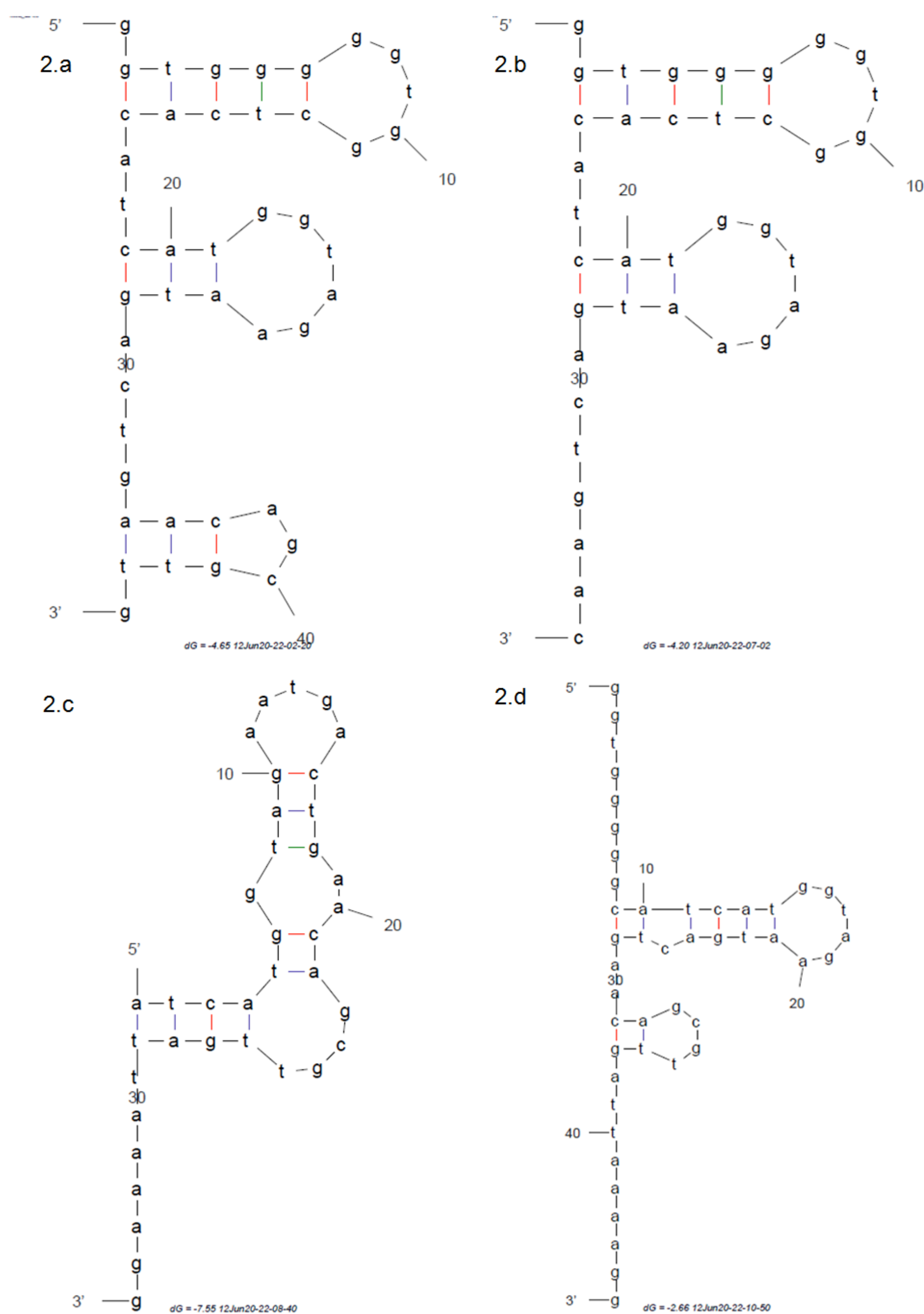

Figure 5.2. Secondary structures modelled using m-fold. The truncated sequences derived from S2 β–conglutin aptamer.

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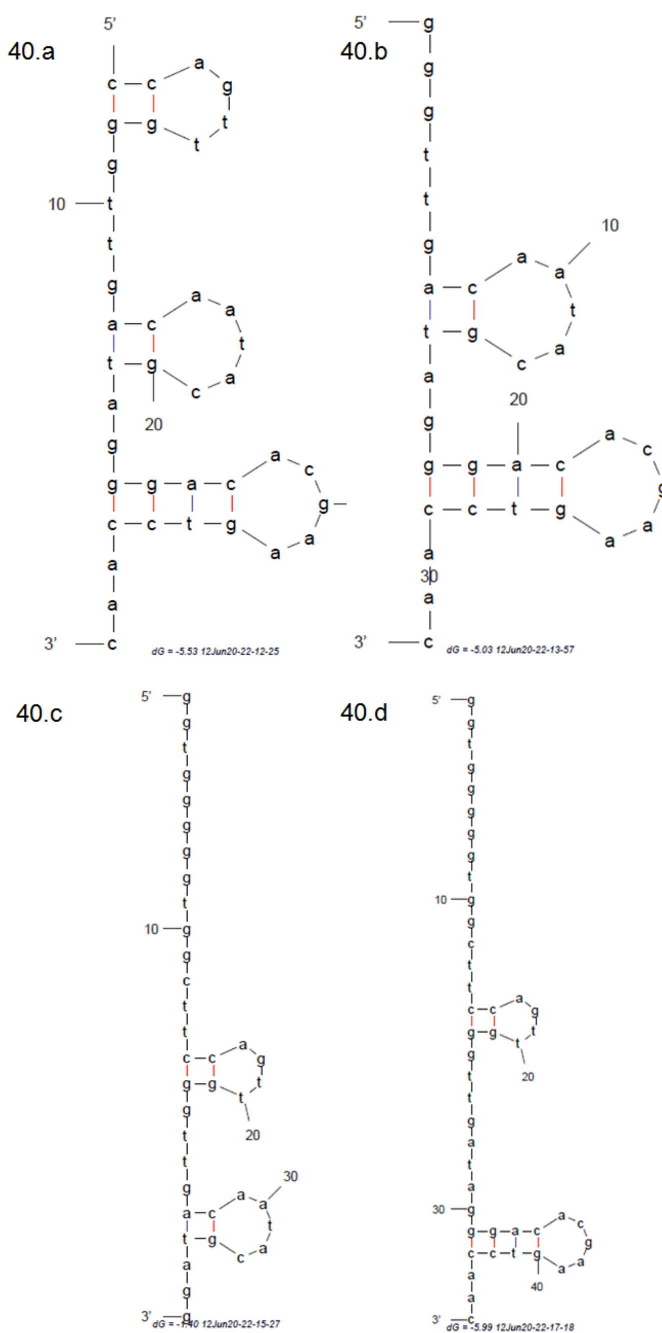

Figure 5.3. Secondary structures modelled using m-fold. The truncated sequences derived from S40 β–conglutin aptamer.

Thus, maintaining the GQ motif, the secondary structure of the aptamer sequence was modelled with the m-fold (Fig. 5.2-3) and the different loops of the secondary structure predicted were removed, resulting in different sequences: S2a, S2b, S2c, S2d, S40a, S40b, S40c, S40d as outlined in Table 5.2.

Table 5.2. Truncated aptamer species (5'-3' direction).

Label	Sequence (from 5' to 3')	Nucleotides	MW ($g \cdot mol^{-1}$)
S2.a	**ggtg ggg gtg g**ct cac atc atg gta gaa tga ctg aac agc gtt g	44	13734
S2.b	**ggtg ggg gtg g**ct cac atc atg gta gaa tga ctg aac	37	11535
S2.c	atc atg gta gaa tga ctg aac agc gtt gat taa aag g	37	11510
S2.d	**ggtg ggg g**c atc atg gta gaa tga ctg aac agc gtt gat taa aag g	46	14408
S40.a	cc a**gt tgg gtt** gac aat acg tag gga cac gaa gtc caa c	39	12051
S40.b	**gg gtt g**ac aat acg tag gga cac gaa gtc caa c	33	10222
S40.c	**ggtg ggg gtg g**ct tcc agt tgg gtt gac aat acg tag g	38	11903
S40.d	**ggtg ggg gtg g**ct tcc agt tgg gtt ga tag gga cac gaa gtc caa c	46	14368

The underlined bases in bold are the consensus GQ motif section.

The binding characteristics of these sequences were analyzed together with the main SGQ motif and its non-truncated counterparts (S2, S40) using SPR on a CM5 Biacore chip. Here the target β-conglutin was attached by amino coupling, and γ-conglutin and gliadin were used as negative controls. In order to check possible cross-reactivity of the truncated species a high amount of DNA was passed over the surface of the prepared chip. According to the technique employed the RU obtained are proportional to the molecular weight of the molecule and to make the responses comparable, the RU obtained were normalized with the corresponding mass of the sequence. As can clearly be seen the highest signal was obtained for the G-Quadruplex structure of 11-mer (Fig. 5.4), and none of the structures predicted using m-fold resulted in aptamers of high affinity.

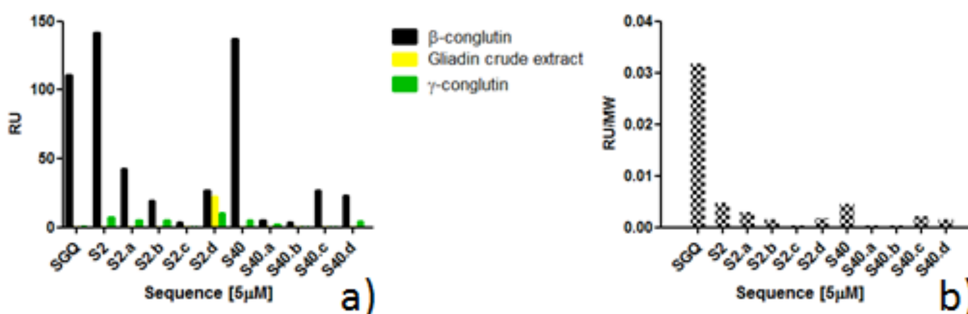

Figure 5.4. Evaluation of different truncated sequences by SPR. a) RU obtained for each sequence showing the interaction with the immobilized target (Channel 1: β-conglutin) and negative controls (Channel 2: γ-conglutin, Channel 3: gliadin). b) RU

reached at the plateau of the association curve normalized by the molecular weight of each molecule in a Biacore CM5 chip with target (β-conglutin).

Cross-reactivity was negligible for the 11-mer sequence as well for the non truncated aptamers (Fig. 5.4b). Binding was observed as compared to the non truncated aptamers, with a considerable improvement in the dissociation constant [21] (Fig. 5.5). The Kd of the truncated GQ-motif SGQ β-conglutin aptamer was determined using a Langmuir model and was vastly improved from 3.60 x 10^{-7} to 5.88 x 10^{-10} M. The good fit to the model was obtained as demonstrated by the χ^2 values of 1.56. [**Note**: The Chi^2 value is a standard statistical measure of the closeness of fit of data to the model used for elucidation of the K_D, where for good fitting to ideal data, χ^2 is of the same order of magnitude as the noise in RU, typically <2].

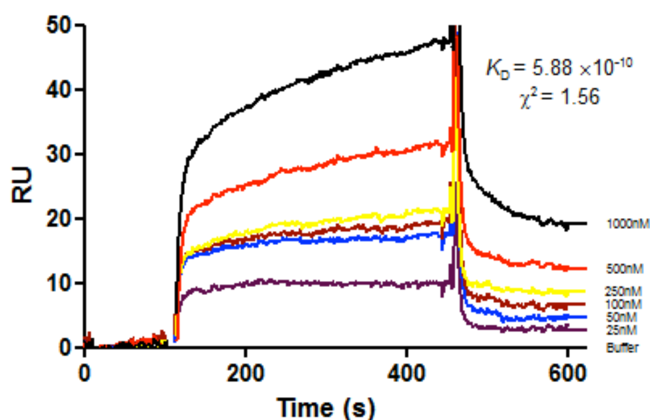

Figure 5.5. SPR Sensorgram obtained for Sequence GQ at 1 µM to 25 nM passed through the CM5 Biacore chip surface showing the interaction with the immobilized target (Channel 1: β-conglutin) and negative controls (Channel 2: γ-conglutin, Channel 3: Gliadin Crude Extract).

5. CONCLUSIONS

The structure of the reported aptamers against the Lup an 1 allergen β-conglutin [21] had been truncated. Through comparing the predicted secondary structures of the aptamers, a hairpin structure with G-rich loop was determined to be the binding motif of these aptamers.

The highest affinity was observed with a truncation resulting in an 11-mer sequence that had an apparent equilibrium dissociation constant (Kd) of 5.88×10^{-10} M. This 11-mer sequence was demonstrated to have high specificity for β-conglutin and showed no cross-reactivity with γ-conglutin or gliadin. Further work will focus on the elucidation of the structure of this truncated 11-mer SGQ aptamer.

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7.SUPORTING INFORMATION

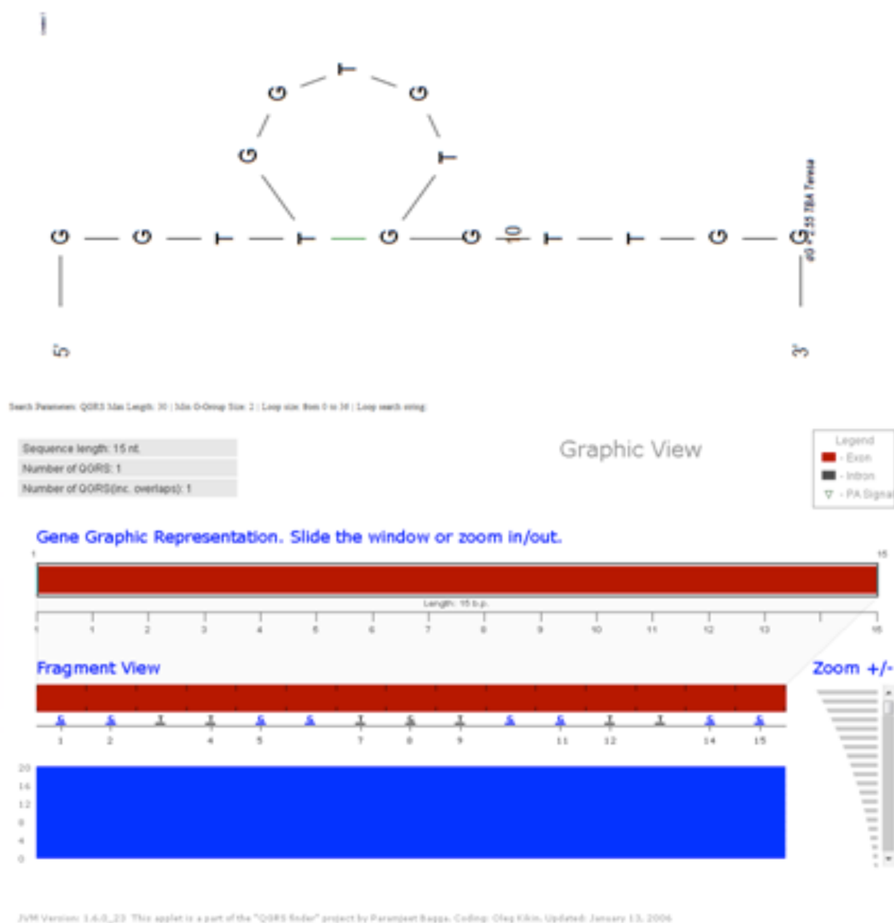

Figure 5.S1. m-fold and GQRS-mapper graph showing the secondary structure, and the probability to find a G-quadruplex motif for TBA.

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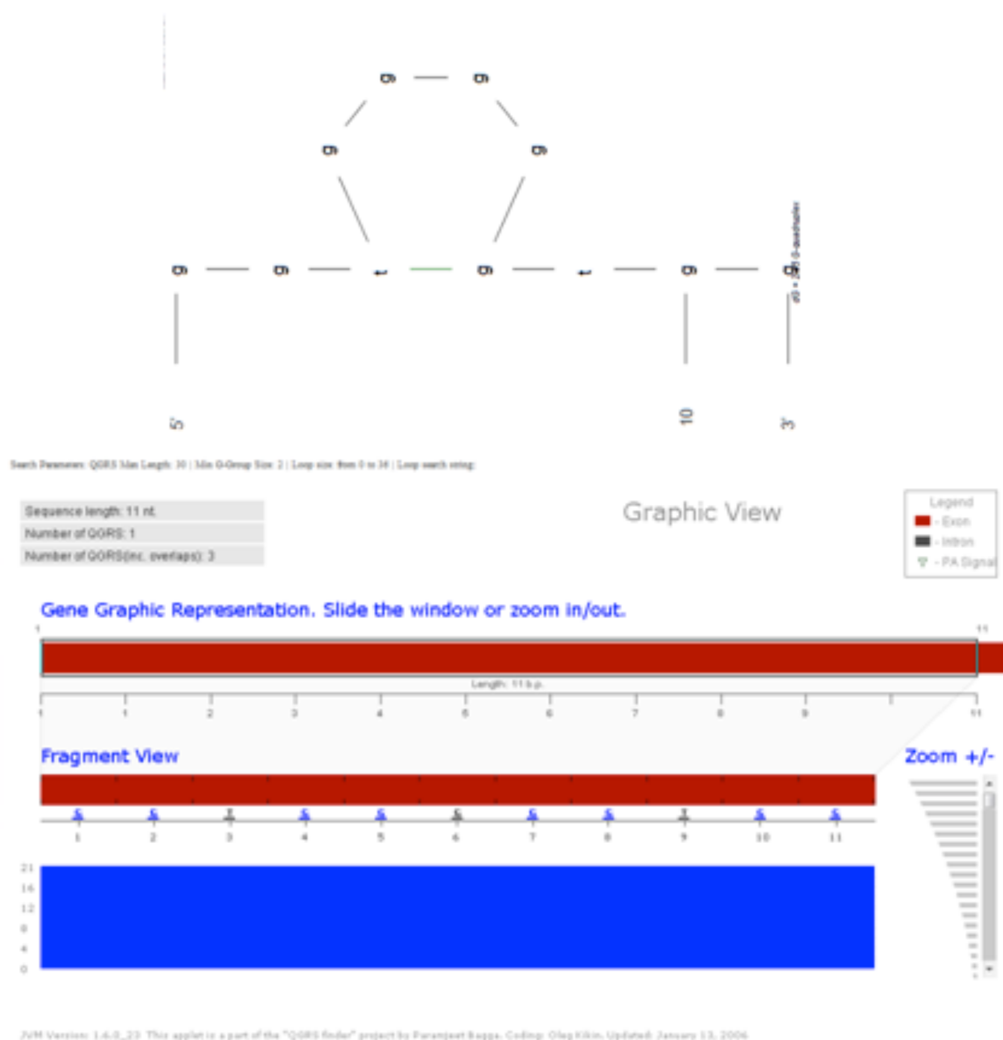

Figure 5.S2. m-fold and GQRS-mapper graph showing the secondary structure, and the probability to find a G-quadruplex motif for SGQ.

CHAPTER 6

STRUCTURE ELUCIDATION OF TRUNCATED 11-mer LUP AN 1 APTAMER

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1. ABSTRACT

There is a clear interest in developing methods to detect the lupin allergen, because of a high number of severe food allergens reactions reported. In order to improve the developed analytical tools for the detection of the lupin allergen β-conglutin, Lup an 1, the structure of the truncated 11-mer Lup an 1 aptamer has been elucidated. Following Reverse Phase HPLC, purification mass spectroscopy studies were carried out by HPLC-TOF, comparing the retentions times from the chromatogram with similar structures and with established G-quadruplex containing structures. The G-rich loop region of this binding motif was further characterized by NMR. This study revels that the 11-mer aptamer possessed a more complex structure than a G-quadruplex due to the presence of a dimeric form of the 11-mer aptamer that has been elucidated by TOF-MS-ES mass spectroscopy, FRET and SPR.

2. INTRODUCTION

Lupin is an herbaceous plant of the leguminous family. This plant belongs to the genus *Lupinus*, which includes more than 450 species. Lupin seeds have been used as human food and animal feed since ancient times [1]. Over the last 10 years, research and developments have demonstrated that lupin products have an added value over numerous bakery products and have some advantages in comparison to other legumes like soy [2]. However after the admission of lupin as a food ingredient, a high number of severe food allergy reactions have been reported [3, 4]. Lupin allergy apparently arises by either primary sensitization [5-7] or clinical cross-reactivity in individuals who are allergic to peanut [4, 8, 9]. These cross-allergic clinical reactions of peanut to other members of the leguminous family, such as soy, peas, beans, and lentils, occurs in about 5% of peanut-allergic patients but were found to be 68% with lupin [10]. Lupin flour and lupin products thus requires mandatory advisory labelling on foodstuff sold in the European Union [11], and recently the International Union of Immunological Societies (IUIS) allergen nomenclature subcommittee designated β -conglutin as the Lup an 1 allergen [12].

Aptamers are artificial receptors that bind to their cognate target with high affinity and specificity and are selected through a Darwinian-like evolution method [13, 14]. Aptamers which are DNA or RNA oligonucleotides that have been screened from a randomly generated population of sequences for their ability to bind a desired molecular target [15]. We have recently reported on the selection of an aptamer against β-conglutin, the lupin subunit that has been identified by the IUIS as the Lup an 1 allergen. This selected aptamer was subsequently truncated from a 93-mer with K_D 360 x 10^{-7} M to an 11-mer with K_D of 5 x 10^{-10} M. This truncated 11-mer is Guanine rich (9Gs in the 11-mer) and is thus expected to fold into G-quadruplex structures, composed of stacked guanine tetrads, which are stabilized by Hoogsteen-type hydrogen bonds between the guanines and by interactions with cations located between the tetrads (Figure 6.1.). In the work reported here, we further probe the elucidation of the structure of this novel short, highly specific and sensitive aptamer using various techniques, including HPLC-TOF, TOF-MS-ES, NMR and Fluorescence (Föster) Resonance Energy Transfer (FRET) studies.

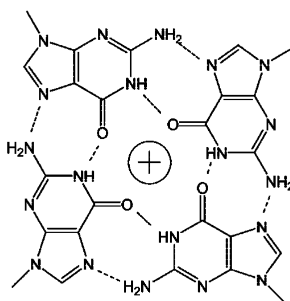


Figure 6.1. G-quartet [16].

Several reports have been published describing the use of chromatographic and mass spectroscopy techniques for the characterisation of aptamers. A recent report describes the characterisation of superparamagnetic nanoparticles conjugated to a fluorescently labelled oligodeoxyribonucleotide by ESI-MS, and analyzed by RP-HPLC chromatography [17]. Another one describes a method for labelling aptamers chemiluminescently with isoluminol isothiocyanate and subsequently the ILITC-labeled aptamers were characterized by HPLC-MS and purified by HPLC [18]. Other authors exploited the basis of RP-HPLC to develop and use quinoline derivatives to provide a general strategy to photo-regulate oligonucleotide activity with improved caging and uncaging efficiency [19]. Finally, a recent report describes the coupling of an acoustic wave biosensor with mass spectrometry, using aptamers for the detection of protein complexes [20].

FRET, is a powerful technique for characterising distance dependent interactions on a molecular scale. FRET is the radiation-free transfer of energy from an excited donor dye to a suitable acceptor dye, a physical process that depends on spectral overlap and correct dipole alignment of the two dyes. When the distance between the two dyes increases the efficiency of energy transfer is markedly decreased [21]. The use of FRET for the elucidation of the aptamer structure has been reported, for example to develop competitive FRET displacement assay with aptamers to detect *E. coli* and determine the location of the putative aptamer binding pocket, by individually testing FRET potential, in each of the secondary loop structures [22]. FRET has also been used to simulate quantum dot-aptamer structure and provide insight into the underlying architecture [23]. FRET could be also used combined with others techniques to analyze aptamer-target interactions. For example de la Faverie et al. combined FRET with FID (Fluorescent intercalator displacement assay) to characterise two synthesised biotin-tagged G4 ligands to elucidate which compound exhibited the best characteristics (the best biotin pyridocarboxamide derivative with high stabilization of an intramolecular quadruplex and excellent duplex-quadruplex specificity) to be used as a target for *in vitro* selection (SELEX) [24]. In more recent studies Yuanboonlim et

al. combined FRET with Circular Dichroism for study the K^+ effect in the stabilisation of the G-quadruplex structure of the aptamer [25].

3. MATERIALS AND METHODS

Reagents. Phosphate Buffered Saline (10 mM phosphate, 138 mM NaCl, and 2.7 mM KCl, pH 7.4), reagents were purchased from Sigma (Barcelona, Spain). Sodium chloride, sodium hydroxide 2 M, hydrochloric acid 6 M, concentrated nitric acid, was purchased from Scharlau Chemie S.A. (Barcelona, Spain). The mass spectra standard calibrators kit was obtained from Per Septive Biosystems. Lyophilized oligonucleotides were synthesized by Ella Biotech GmbH (Martinsried, Germany). All solutions were prepared in high purity water obtained from a Milli-Q RG system (Barcelona, Spain).

Reverse Phase HPLC. The different oligonucleotides (dissolved in water), were purified by reverse phase HPLC on a Dionex HPLC systems using a Supercosil Column CC-18, 25 cm x 4.6 mm with a bead size of 5 µm. The column was equilibrated with 50 mM ammonium acetate, pH 6.5. (Eluent A) and eluted with a linear constant gradient during 15 min from 0 to 100% of 50 mM ammonium acetate in 50% CH_3CN, pH 6.5 (Eluent B) at a mobile phase flow of 1 mL/min. The absorbance of each of the fractions was measured at 260 nm and the isolated fractions were collected using an autosampler and vacuum dried for further analysis.

NMR Experiments. ^{1}H-NMR spectra were acquired at 500 MHz using a Varian Unity Inova-500 NMR spectrometer. HPLC-purified and lyophilized SGQ β-conglutin aptamer was diluted in 90% v/v H_2O/10% v/v D_2O to 2 mM. One-dimensional spectra were acquired by double pulsed field gradient selective excitation for H_2O suppression at 22°C before/after subsequent additions of 10, 20, 30, 40 and 50 mM KCl.

HPLC-TOF. A 1200 Series HPLC coupled to a 6410 Time of Flight LC/MS (Agilent Technologies) was used, with a XDB-C18 4.6x50 mm, 1.8 µm (Agilent Technologies). An autsampler was used for injections of 10 µL. The column was equilibrated with 5 mM ammonium acetate, pH 8. (Eluent A) and eluted with a linear constant gradient during 15 min from 5 to 100% of 50 mM ammonium acetate in 50% MeOH, pH 8 (Eluent B) at a mobile phase flow of 0.4 mL/min. The absorbance of the fractions was measured at 260 nm. The acquisition mode of the ion source was set to detect negative ions with a range from 100 m/z to 3000 m/z. Using a gas temperature of 350ºC in a flow 9 L/min, the nebulizer used 35 psi in the ionisation source. The reference masses used 1033,988109 and 980,016375.

Mass spectrometry. Samples (10 µM in water) were diluted to 50 % acetonitrile/0.1 % formic acid, and analysed by Z-spray nanoelectrospray ionisation MS using a quadrupole-IMS-orthogonal time-of-flight MS (Synapt HDMS, Waters UK Ltd., Manchester, U.K.) using gold coated nanospray vials (Waters UK Ltd., Manchester, U.K.). The MS was operated in positive TOF mode using a capillary voltage of 1.5 kV,

cone voltage of 20 V, nanoelectrospray nitrogen gas pressure of 0.1 bar, backing pressure of 2.47 mbar and a trap bias of 4 V. The source and desolvation temperatures were set at 80°C and 150°C, respectively. During TOF-MS acquisition, Argon was used as the buffer gas, at a pressure of 4.0×10^{-3} mbar in the trap and transfer regions and 4.0×10^{-4} mbar in the IMS cell. Mass calibration was performed by a separate injection of sodium iodide at a concentration of 2 µg/ µl. Data processing was performed using the MassLynx v4.1 suite of software supplied with the mass spectrometer.

FRET detection experiments. All fluorescent measurements were performed in a Cary Fluorimeter at 22ºC constant temperature. The labelled oligonucleotides were mixed and incubated during 30 min at 22ºC in PBS. An Alexa Fluor 488 and Alexa Fluor 555 FRET couple was used. FRET occurs as the Alexa Fluor 488 is excited at 492 nm and emits at 519 nm, exciting the Alexa Fluor 555 (excited at 535 nm), which emits at 565 nm. Thus if FRET occuring, there should be emission at 568 nm following excitation at 492 nm. Changes in FRET decay were demonstrated through a variable emission scan (500–700 nm) at a fixed excitation wavelength (492 nm).

Surface plasmon resonance (SPR) analysis

In the case of immobilizing aptamer on the surface, thiolated truncated sequences of interest were immobilized on a gold sensor chip [26]. After immobilization of the sequence, the chip was blocked via injection of an excess of mercaptohexanol. A range of protein (β-conglutin, γ-conglutin and Gliadin) concentrations were mixed with 10 µM of DNA and incubated in 10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, pH 7.4 in an eppendorf for 30 min at 22ºC. Subsequently, 10 µL of this mixture was injected at a flow rate of 2 µl/min for 5 min followed by 5 min dissociation time. The surface was regenerated by 3 injections of 10 µL NaOH at 20 mM.

4. RESULTS AND DISCUSSION

In order to obtain, high quality data it is essential that the molecule under study is in a highly purified form and to this end the 11-mer truncated β-conglutin aptamer was purified using Reverse Phase HPLC. The retention time of the 11-mer was compared to that of 15-mer, 17-mer and 21-mer (SA, SB and SC) random sequences and unusual behaviour was observed with the 11-mer having a longer retention time than the longer and higher molecular weight oligomers (Table 6.1). We postulated that this extended retention time could be due to the presence of a G-quadruplex as had been predicted using the GQRS-mapper.

Table 6.1. Sequences used in Reverse Phase HPLC (5'-3' direction).

Label	Sequence (from 5' to 3')	Nucleotides	MW ($g \cdot mol^{-1}$)	Retention time (min)
SGQ	ggt ggg ggt gg	11	3510	11,317
SA	gca cgt agg caa cta	15	4586	9,758
SB	gtt ttc cca gtc acg ac	17	5121	9,984
SC	gac gcg ttg gag ctg gta tca	21	6502	9,733

Furthermore, as can be seen in Figure 6.2, 3 peaks, a major one and two minor, almost overlapping ones, were observed.

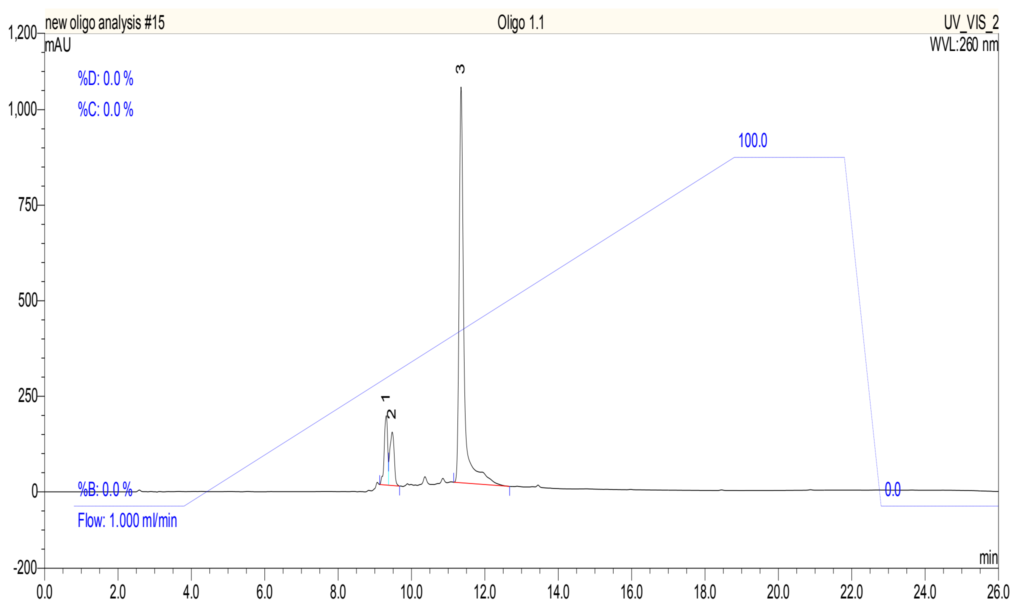
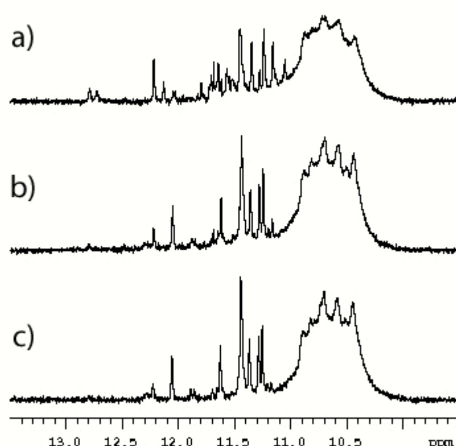


Figure 6.2. Reverse Phase HPLC showing the chromatogram obtained for the 11-mer Oligonucleotide sequence 5'-GGTGGGGGTGG-3', MW 3509 g/mol, with Rt 11,317 min.

To probe the presence of a G-quadruplex structure in the SGQ β-conglutin aptamer, a DPFGSE spectra for water suppression (double pulsed field gradient selective excitation) was carried out at 22 ºC by KCl titration at 0, 10, 20, 30, 40, 50 mM. The structure was stabilized at 40 mM of K^+ cations dissolved in ultrapure water. The experiment was repeated in the presence of 0 mM, 20 mM and 40 mM KCl added at 22 ºC (Fig. 6.3). The signals between 11 and 12 ppm signify G-quadruplex imino's. At the lowest KCl concentration at least two and possibly three different forms of quadruplex are co-populated (there are about 18 discernible peaks). At 40 mM KCl only a single form of quadruplex is populated, while the population of the alternative quadruplex forms decreases at higher concentrations of KCl. The six imino proton chemical shifts between 11.1 ppm and 12.2 ppm resonate in the region characteristic of guanine imino protons involved in GGGG tetrad formation [27]. The effect of $MgCl_2$ was also carried out using titration studies, but no particular effects were observed. Furthermore the shifting position of the imino protons of the guanine and thymine nucleotides in the NMR spectra obtained for the GQ sequence at 40 mM KCl at 22 ºC by the resonance shows similarities to the guanine quadruplex described by Kuryavyi et al. [28].



Figuere 6.3. NMR spectrum of the 11-mer SGQ β-conglutin aptamer with sequence: GGTGGGGGTGG. ^{1}H-NMR spectra were acquired at 500 MHz from HPLC-purified and lyophilized Sequence GQ diluted in 90:10% v/v H_2O/D_2O. The spectra were acquired at 22 °C in the absence (a) or in the presence of KCl at 20 mM (b) and 40 mM.

The NMR results clearly indicate a G-quadruplex in the presence of K^+ and future work will probe the role of the β-conglutin target as chaperone for quadruplex formation. As the NMR studies demonstrated the presence of a quadruplex the unexpected longer retention in RP/HPLC was attributed to presumably be due to the presence of this complex structure.

To probe this hypothesis, the 11-mer β-conglutin aptamer was analysed and compared to closely related sequences, as well as to other sequences known to form G-Quadruplex structures, as detailed in Table 6.2. Sequences SGQ-bio, S1 are very similar to the 11-mer truncated SGQ β -conglutin aptamer with the former being appended by a TEG-Biotin tail and the later with three Thymine/Adenine bases, whilst the S3 sequence lacks a terminal G base, effectively reducing the probability of tetrad formation. The TBA is a 15-mer aptamer known to form a G-Quartet and being of similar length to the SGQ β-conglutin aptamer. If the increased retention time observed was due to the presence of the quadruplex then both TBA and SGQ would be expected to behave similarly. Additionally S3, a 22-mer not predicted to form G-Quadruplex structures was also analysed to test if the 11-mer had coupled with another 11-mer in a linear-type fashion.

Table 6.2. Aptamer truncated sequence, its derivatives and controls (5'-3' direction).

Label	Sequence (from 5' to 3')	Nucleotides	MW (g·mol^{-1})	Retention time (min)
SGQ	ggt ggg ggt gg	11	3510	9.81
SGQ-bio	ggt ggg ggt gg-TEG-biotin	11	4080	10.04
S1	tat ggt ggg ggt gga ta	17	5362	9.49
S2	ggt ggg ggt gt	11	3485	1.41
S3	gtg tgt gtg tgtg tgt gtg tgt	22	6905	1.36
TBA	ggt tgg tgt ggt tgg	15	4727	1.38

Interestingly, again the results revealed that a structure more complex than a G-quartet was present. The SGQ β-conglutin aptamer, SGQ-bio and S1 sequences, which all contained the complete 11-mer sequence, behaved similarly with long retention times. The S2 sequence, which due to being Guanine-rich (8 Guanines) can be expected to be structured, even though the replacement of the terminal G with a T prevents the formation of a quadruplex. However, the retention time was vastly reduced and more or less equal to that of the unstructured 22-mer, leading us to believe that the lengthy retention time was indeed due to the G-Quadruplex present in the 11-mer. However, the TBA, known to form a G-Quadruplex, showed almost the same retention behaviour as the S2 sequence, clearly demonstrating that the SGQ forms a further more complex structure, which results in the longer retention time observed.

To further probe the structure of the 11-mer, mass spectrometry MS was used. MS is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules, such as peptides and other chemical compounds. MS works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios, and if this ratios results in the double mass of the molecular weight expected, could be a signal of molecular dimer formation.

In a first approach HPLC-TOF was carried out, using the fraction that eluted at 9.81 min as can be seen in Figure 6.2, the mass spectra obtained for the eluted peaks of the HPLC show the corresponding masses of the monomerical molecule divided by charge, ranging from 2 to 4, apart from one peak, corresponding to a dimerized form (Fig. 6.4).

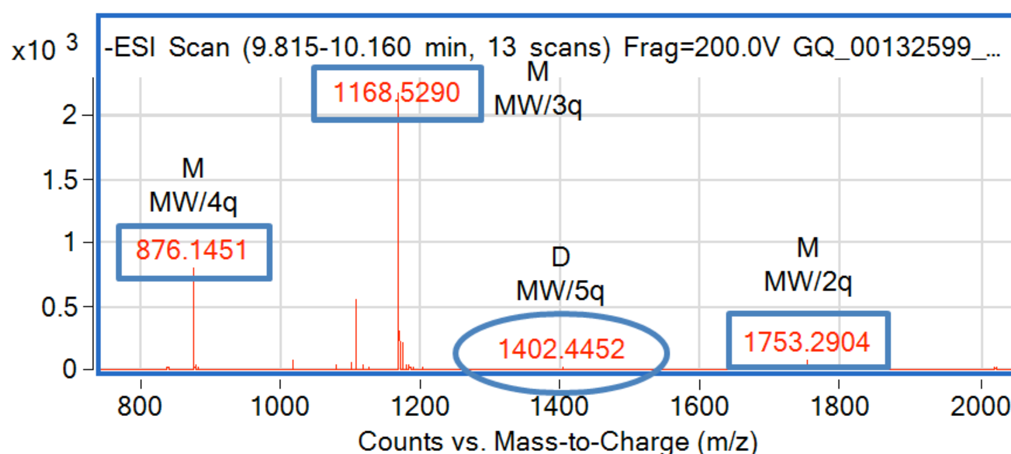


Figure 6.4. Electrospray Ionization-Scan obtained for the chromatogram eluted peack at 9.81 min, showing the peacks of the mass spectra for the monomer (M) and dimer (D). MW/Xq means the molecular weight divided by the corresponding "*X*" charge.

The results indicate the presence of a dimer, but it was feared that the ionisation conditions themselves may have provoked dimer formation, and to this end, the use of nanoelectrospray ionisation coupled to a quadrupole-IMS-orthogonal time-of-flight MS was used, as this technique produces softer ionization conditions. For this study, the three different peaks obtained during RP-HPLC purification were collected using an auto sampler and vacuum dried for analysis using TOF-MS-ES. The results obtained with the first two peaks (1 and 2) were attributed to sample impurities and uncompleted sequences coming from the synthesis process, whilst the third main peak with a retention time 11.3 min was observed to have a mass of 7018.03±0.76 g mol^{-1}, indicating the presence of a dimeric form of the SGQ β-conglutin aptamer (Fig. 6.5).

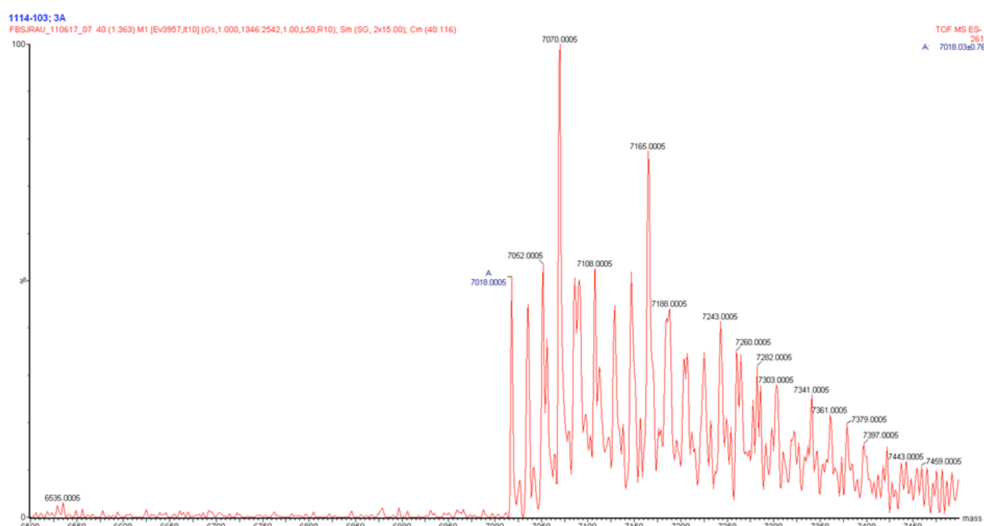


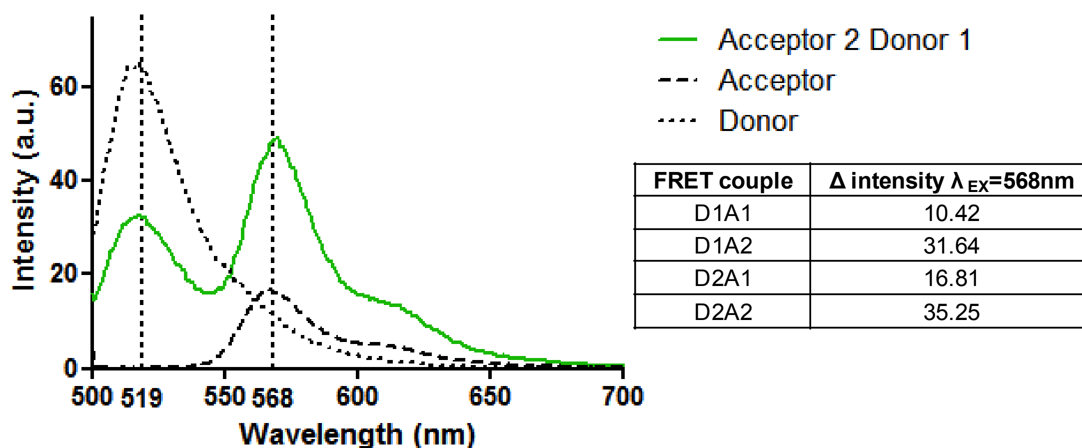
Figure 6.5. Mass Spectra obtained by TOF-MS-ES for the truncated SGQ β-conglutin aptamer showing the parent ion with a mass 7018.03±0.76 g mol^{-1}.

Whilst the results seem to confirm the existence of 11-mer aptamer as a dimer of G-Quadruplexes, there is still a minor possibility that the ionization conditions imposed during analysis may actually be provoking the dimerization and a further technique exploiting FRET was studied to confirm the presence of a dimer. The 11-mer was flanked on either the 5' or 3'-end with the Alexa Fluor 488 donor (λ_{EX}=492nm; λ_{EM}=519nm), as well as being flanked on either the 5' or 3'-end with the Alexa Fluor 555 acceptor (λ_{EX}=553nm; λ_{EM}=568nm), as outlined in Table 6.3, and depicted in Figure 6.6.

Table 6.3. FRET Probes.

Label	Sequence	Excitation (λ nm)	Emission (λ nm)
Donor 1 (D1)	5'- ggt ggg ggt gg -AF488-3'	492	519
Donor 2 (D2)	5'-AF488- ggt ggg ggt gg -3'	492	519
Acceptor 1 (A1)	5'-BIOTIN- ggt ggg ggt gg -AF555-3'	553	568
Acceptor 2 (A2)	5'-AF555- ggt ggg ggt gg -BIOTIN-3'	553	568

FRET takes place as the Alexa Fluor 488 is excited at 492 nm and emits at 519 nm, exciting then the Alexa Fluor 555 (excited at 553 nm), which emits at 565 nm. Thus if FRET occurs, there should be emission at 568 nm following the excitation at 492 nm [21].



FRET couple	Δ intensity λ_{EX}=568nm
D1A1	10.42
D1A2	31.64
D2A1	16.81
D2A2	35.25

Figure 6.6. FRET effect produced by the combination of donor 1 and acceptor 2. Summary of increments in intensity at 568 nm for all the different couples of donor and acceptor.

Using a buffer containing 40 mM KCl, which favours and stabilizes quadruplex formation, a series of experiments were carried out combining different FRET pairs. Firstly to confirm that a dimeric structure does indeed form and secondly, to elucidate what is the favoured orientation of the dimer (5'-3': 5'-3' vs 5'-3': 3'-5'), and the highest FRET is observed for the couple D1A2 and D2A2, indicative of a favoured 5'-3': 5'-3'parallel interaction. Interestingly, when these experiments were repeated in the absence of K^+ the FRET effect was still produced and higher for the same couple D1A2.

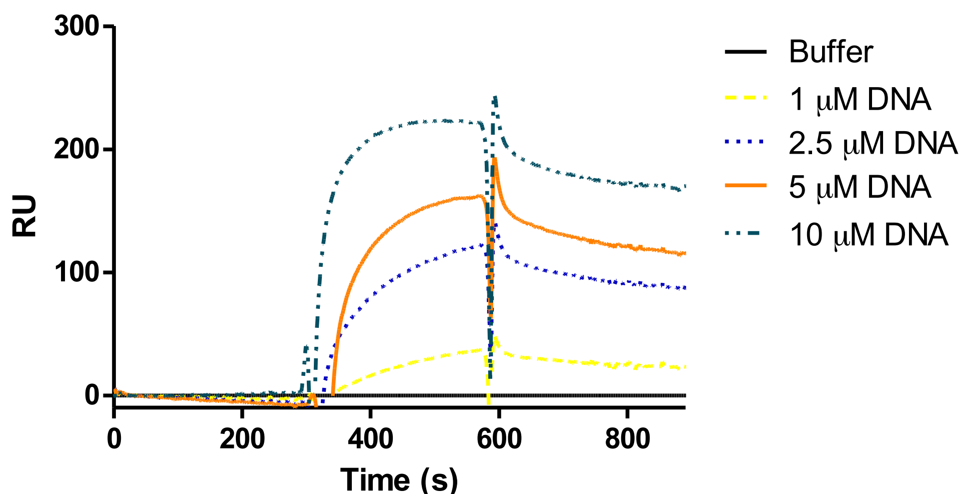


Figure 6.7. SPR figure of the SGQ β-conglutin aptamer showing the interation in SPR between dimmer forms.

Finally to visualise the interaction of the SGQ β-conglutin aptamer during dimerization, the aptamer was immobilized on a gold Biacore Chip and different concentrations of the SGQ β-conglutin aptamer were passed the chip surface to show the interaction. As it is possible to see in Figure 6.7, the SGQ β-conglutin aptamer interacts with itself, and as much we increased the aptamer concentration, more resonance units (RU) are obtained demonstrating the aptamer dimer formation. For an immobilization level of aptamer in surface obtained 685,6 RU, this chip surface became saturated at 10 μM of SGQ β-conglutin aptamer, thus demonstrating again the formation of the dimer.

5. CONCLUSION

Using the truncated 11-mer SGQ β -conglutin binding aptamer, several techniques were used to elucidate its' structure. RP-HPLC was used to purify the aptamer prior to analysis, and the retention time observed was not as expected suggesting some complex structuring, which was presumed to be due to the presence of a G-quadruplex, which was predicted by the GQRS-mapper and experimentally demonstrated using NMR. HPLC-TOF studies were thus carried out, comparing the retention times with similar structures and with established G-quadruplex containing structures, clearly revealing that the 11-mer aptamer possessed a more complex structure than a G-quadruplex. Consequently TOF-MS-ES was carried out indicating the presence of a dimeric form of the 11-mer aptamer. Finally, FRET unequivocally demonstrated the presence of a dimeric aptamer in a 5'-3'; 5'-3' parallel orientation, and the interaction between dimers was also shown using SPR.

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CHAPTER 7

Conclusions and Future Work

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CONCLUSIONS AND FUTURE WORK

The doctoral dissertation presented represents a significant contribution to the state of the art of lupin detection – directly lending itself to *in situ*, facile and inexpensive analysis.

The first part of the thesis focused on aptamer target preparation, starting from lupin seeds that had been milled and deffated. Protocols for extraction and selective isolation of lupine α, β, γ, and δ proteins were elucidated exploiting isoelectric precipitation, salt extraction and ultracentrifugation and varying parameters such as duration and speed of centrifugation or types of salts and salt concentrations. Furthermore, the isolated proteins were characterised using reducing and non-reducing electrophoresis, western blotting, and peptide mass fingerprinting. All these techniques confirmed the purity of each of the conglutin subunits. The protocol elucidated can also be used to selectively extract and purify specific globulin proteins from commercial foodstuffs for analysis. Furthermore, the isolated proteins can be used to elucidate information regarding toxicity of each protein by looking at their interaction with the IgE antibodies found in the serum of individuals allergic to lupin.

In the second part of the thesis SELEX, using the isolated and purified β-conglutin target conjugated to magnetic beads, was achieved. The final enriched DNA candidate aptamers were cloned and sequenced, and using different softwares, consensus motifs were identified, and these consensus motifs were used to detect the Lup an 1 allergen and demonstrated to be of high affinity and specificity, capable of distinguishing it from other possible proteins present in flour, e.g. gliadin, or other globulin proteins present in lupin such as γ -conglutin. Moreover, the secondary structures of the obtained sequences were predicted and evaluated using the GQRS-Mapper, obtaining a high G-score highly suggesting the presence of G-quadruplexes.

Once the functionality of the obtained aptamer had been demonstrated, truncation studies were carried out to optimise its' structure. The specific bases not believed to be essential for binding were removed and the effect on affinity was determined in order to identify the essential bases. The aim of this is to have an aptamer where only the essential bases are present, and truncation often improves the affinity of the aptamer, or maintains it, whilst resulting in shorter sequences, inherently of a lower cost. The truncated lupin aptamer presents one of the shortest binding motif reported to date, being just 11 bases in length and had a low dissociation constant of $5{,}88 \times 10^{-10}$ M. This 11-mer sequence was demonstrated to have high specificity for β-conglutin and showed no cross-reactivity with γ-conglutin or gliadin.

The structure of the truncated 11-mer Lup an 1 aptamer was elucidated. Through comparing the predicted secondary structures of the aptamers, a hairpin structure with G-rich loop was determined to be the binding motif of these aptamers. The binding G-rich region resulted in a G-quadruplex structure following characterisation by NMR. Following Reverse Phase HPLC purification, mass spectroscopy studies were carried out using HPLC-TOF. Then comparing the retention times with similar structures and with established G-quadruplex aptamer containing structures, reveals that the 11-mer aptamer possessed a more complex structure than just a G-quadruplex. Finally, the presence of a dimeric form in this 11-mer aptamer was elucidated using TOF-MS-ES mass spectroscopy and FRET.

Furthermore the applicability of aptamers was demonstrated using a competitive ELONA assay, revealing acceptable limits of detection in the nanomolar range. Additionally, an apta-PCR assay, which would facilitate extremely low detection limits was developed, where the aptamers themselves are used as both biorecognition and reporter molecules was developed. Apta-PCR improved the detection limit attainable by ELONA assay 25,000-fold, achieving the ultrasensitive detection of the Lup an 1 allergen in the picomolar scale.

Overall, the outcomes of this thesis have contributed significantly to the development of an alternative biorecognition element for the anaphylactic allergen of Lupin β–conglutin (Lup an 1). Furthermore this novel biorecognition molecule, the aptamer, has been optimised and the structure elucidated. Finally the aptamer was applied to different assays for the specific detection of β–conglutin.

Future perspectives will be addressed to the development of others assays and aptasensor platforms for the detection of the β–conglutin allergen. Finally, the most easy to use assay will be applied for real sample analysis, and the reliability and cost for the final assay validation will be assessed.

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