Leguminous Lectins Bind Non-specifically to DNA

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I affirm, that I carried out the present PhD-thesis independently and that all the literature and techniques that I used were as referenced.

Otilia Vieira Breitwieser

Cologne, ___ / ___ / ___

This work was carried out from January 2000 to September 2003 at the Institute for Genetics of the University of Cologne under the supervision of Professor Dr. Walter Doerfler.

to

my parents

FIGURES

TABLES

ABREVIATIONS

A	adenine
Abs	absorbance
ATP	adenosidetriphosphat
BHK	baby hamster kidney
Bp	base par
BSA	bovine serum albumine
C	cytosine
CD	circular dichroism
ConA	Concanavalin A
ConBn	lectin of *Canavalia bonariensis*
ConBr	lectin of *Canavalia brasiliensis*
ConG	lectin of *Canavalia grandiflora*
ConM	lectin of *Canavalia maritima*
Ci	Curie
cpm	counts per minute
dest.	destilled
Dviol	lectin of *Dioclea violacea*
Dvirg	lectin of *Dioclea virgata*
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift
EtdBr	ethidium bromide
EtOH	ethanol
FISH	Fluorescence *in situ*-hybridization
g	gram
Gal	galactose
Glu	glucose
GFP	green fluorescence protein
h	hours

kDa	kilodaltons
L	liter
LcA	*Lens culinaris* lectin
M	mol
min	minute
mM	milimolar
mRNA	messenger RNA
µg	microgram
µl	microliter
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
pEGFP-C1	enhanced green fluorescent protein plasmid vector – C-terminus (Clontech)
PBS	phosphate buffered saline
PHA-E	lectin of *Phaseolus vulgaris* (Erythroagglutinine)
PHA-L	lectin of *Phaseolus vulgaris* (Leukogglutinine)
RNA	ribonucleic acid
rpm	rotations per minute
SBA	soya bean agglutinine
SDS	sodium dodecyl sulfate
TBE	tris-borate-EDTA
TE-buffer	tris-EDTA-buffer
UV	ultraviolet light
VVLB4	*Vicia villosa* isolectin 4

1. INTRODUCTION

1. 1 Protein-DNA Interactions

Protein-DNA interactions are one of the most fundamental interactions between macromolecules in living organisms. Almost all functions of DNA are mediated by the participation of proteins (Bloomfield et al, 2000; Garvie and Wolberger, 2001; Borukov and Nudler, 2003). Regulatory protein-DNA interactions are responsible for the proper control of gene expression by the recognition of specific DNA sequences and structures. The temporal and local regulation of DNA-replication is highly dependent on the activities of proteins (Neurath and Petterson, 1998; Benos et al., 2002). Enzymatic protein-DNA interactions are required for transcription, replication and repair of DNA (Vassylyev and Morikawa, 1997; Borukov and Nudler, 2003). The structure and conformation of DNA are also controlled by the participation of proteins. The study of protein-DNA interactions is fundamental for the understanding of growth, development, differentiation, evolution and disease (Rhodes and Burly, 1997; Garvie and Wolberger, 2001). The experimental applications of protein-DNA interactions facilitated most of the advances in molecular biology research (Fairall et al., 2000). Protein-DNA-interactions may be analyzed at three major levels: (i) at the level of **DNA**, (ii) at the level of **protein** and (iii) at the level of **protein-DNA-complexes** (Bloomfield et al., 2000). When DNA is the major focus, binding sequences, conformations and structures of the DNA, required for the binding are investigated. At the protein level, the amino acids involved, the contact-surface and the recognition motives have to be identified. At the level of the protein-DNA-complex, binding conditions, kinetics of association and the binding mechanism can be characterized (Kabanow et al., 1998). The study of the complex is greatly facilitated when the biochemical properties of each component are known.

1. 1. 1 Interactions of DNA with other molecules

DNA is a linear polymer consisting of repeated nucleotide units. The double helix structure of the DNA is a consequence of the arrangement of its components: the heterocyclic base (A, C, G and T), the sugar 2-D-deoxyribose and the phosphate ester. While the complementary relationship of the bases inside the DNA-helix encodes the genetic information, the sugar-phosphate backbone forms the exterior of the DNA double helix (Watson and Crick,

1953). The properties of these components separately and in conjunction as part of the DNA-helix have to be considered for the understanding of how DNA-molecules interact with their environment (Privé et al., 1991). The most fundamental interaction of DNA with its environment is the binding of water and ions (Schwabe, 1997). Water is the major solvent component for DNA in all biologically relevant processes (Westhof, 1988). The relative humidity, or water content, influences the structure of the DNA helix (Berman, 1994). Crystallographic and physical chemical studies have shown that local hydration and the surface water-network of the DNA mediate surface contacts for many protein-DNA interactions (Schwabe, 1997). DNA molecules are highly charged polyanions with one negative charge per phosphate. Therefore, DNA interacts strongly with positively charged ions. The binding of DNA to cations, specially some divalent ions such as Mg^{2+}, stabilizes the helix conformation by shielding the negative charges of the phosphodiester backbone (Xu and Bremer, 1997; Li et al., 1998). The polyelectrolyte behavior of the DNA is therefore important for the binding of charged protein ligands, for the transition of helix conformations (A-, B- and Z-forms of DNA) and for DNA condensation. The ionic binding of a protein to DNA can also be mediated by the coordination of cations to the double helix. Sequence-specific binding of Mg^{2+} and Ca^{2+} to the major and minor grooves of DNA B-form has been reported by crystallization studies (Chiu and Dickerson, 2000). The sites binding to metal ions are distributed in all components of the DNA. Phosphate-oxygen atoms bind to metal ions through salt linkages. Sugar hydroxyl groups bind to alkali metals and alkaline earths, but not to transition metals. Nitrogen atoms of the purine or pyrimidine bases bind to metal ions and specially to transition metals.

1. 1. 2 Factors involved in protein-DNA binding

The interactions that mediate protein-DNA binding can be grouped in three major categories: electrostatic, hydrogen bonding and hydrophobic. Electrostatic interactions result from the binding of the negatively charged groups of DNA by a positively charged site of the protein (Bloomfield et al., 2000). Usually, this kind of protein-DNA binding induces the release of small ions into the surrounding solvent, as each binding partner changes the binding to the small ions against the binding to each other. Therefore, the addition of salt to the solvent influences the displacement of protein or DNA-bound ions to the bulk solution.

Protein-DNA interactions mediated by hydrogen bonding are often related to the availability of donors and acceptor groups in the double helix (Garvie and Wolberger, 2001). Base paaring blocks some of the hydrogen bonding positions available to distinguish one base

sequence from another. The remaining hydrogen bond donors and acceptors are available in the minor or minor groove (Seeman et al., 1976; Steitz, 1990). Each base pair offers a distinct combination of acceptor and donor groups. For the recognition of DNA by proteins, a number of amino acid side chains can serve as hydrogen bonding acceptors or donors (Sauer et al., 1990). The amino acids more frequently involved in hydrogen bonding in protein-DNA interactions are Gln, Glu, Ser, Lys, Asn, Arg, Thr, His and Cys (Bloomfield et al., 2000). The formation of these hydrogen bonds requires the proper positioning of the involved amino acids to the major or minor groove (Church et al., 1977). In the B-form of DNA, for example, the major groove can accommodate protein structural motives like α-helices and β-sheets, also called, β-strands (Tateno et al., 1997). Two antiparallel β-sheets can interact with the DNA in the major or minor grooves (Carter and Kraut, 1974, Church et al., 1977). This structural recognition motif is used, e.g., for the binding of *arc*- or *met*-repressors, for the TATA binding protein, and for HU-proteins (Breg et al., 1990; Tanaka et al., 1984; Riddihougth, 1994; Rauman et al., 1994; Nikolov et al., 1996; Rice, 1997; Patikoglou et al., 1999). Major groove recognition by three β-sheet strands is the DNA-binding motif of the Tn916 integrase (Wojciak et al., 1999; Connolly et al., 2000). A combination of defined secondary structures of proteins like α-helix or β-sheet and loops are also widely used as recognition motives (Garvie and Wolberger, 2001). The loop-sheet-helix motif found in the tumor suppressor protein p53 recognizes specific features in the major and minor grooves of its binding sequence (Cho et al., 1994). The participation of β-sheets and loops in DNA recognition is particularly well exemplified by the binding motif of the transcription factor NFkB (Ghosh et al., 1995). This DNA-binding protein abounds in β-sheet strands, and the loops are positioned in the structure to connect the β-sheets (Müller et al., 1995). An interesting aspect of the NFkB structure is the use of loops, which interact with the major groove and the phosphate backbone.

1. 1. 3 The formation of protein-DNA complexes

The investigation of the protein-DNA interactions requires the characterization of the complexes in solution. Environmental parameters like temperature, ionic strength, pH and concentration of the each binding partner have marked influence on complex formation (Fairrall et al., 2000). There are a number of different methods to analyze the formed complexes quantitatively and qualitatively.

Non-denaturing gel electrophoresis has been widely applied for the detection of protein-DNA-complexes. The binding of RNA-polymerase to DNA or the histone-DNA-complexes have

been carried out with this technique (Chelin and Geiduschek, 1979, Klevan et al., 1978). In the early 1980s, many investigations have shown that DNA-bound regulatory proteins, e.g. the *lac*-repressor, could be detected as discrete bands in gel electrophoresis (Gardner and Revzin, 1981, Fried and Crothers, 1981). Since then, the Electrophoretic Mobility Shift Assay (EMSA) has become standard for the characterization of protein-DNA interactions. EMSA provides a powerful method, because of it is easy to use, versatile and is very sensitive (Gerstle and Fried, 1993; Fried and Daugherty, 1998). This assay has the distinct advantage that it can be used to analyze new protein-DNA interactions, even if no information about the parameters required for binding is available. EMSA can also be applied for the estimation of binding kinetics, association/dissociation constants and stoichiometric relationships (Laniel et al., 2001).

1. 2 Lectins

1. 2. 1 General aspects

Lectins are a heterogeneous class of proteins that bind specifically and reversibly to carbohydrates (Sharon and Lis, 1989). Lectins can recognize mono-, oligo- or polysaccharides, as well as glycoconjugates and thus recognize glycoproteins or glycolipids, e.g. on the surface of cells (Sharon and Lis, 1995). The group of multivalent lectins carries two or more carbohydrate binding domains. These lectins are, therefore, able to agglutinate cells or to precipitate glycoconjugates (Reeke and Becker, 1988). Because of their agglutination activities, lectins have been originally described as **cell agglutinins**. The term **lectin** (from the Latin *legere*, meaning to select or to choose) has been introduced by Boyd to designate a broader range of activities involving selectivity for specific carbohydrates (Boyd, 1954; 1963). To be considered as a **lectin** a given protein or glycoprotein must fulfill three requirements: (i) it binds to carbohydrate; (ii) it is distinct from immunoglobulins and (iii) it does not biochemically modify the carbohydrate which it binds (Goldstein et al., 1980; Kocourek and Horejsi, 1983; Gabius, 1997; Rüdiger and Gabius, 2002). Furthermore, lectins are distinguished from saccharide transport proteins and from “sensors” for free mono- or disaccharides, which act in chemotaxis or in operon systems (Kocourek and Horejsi, 1983). Fig. 1 shows a representation of a lectin binding to carbohydrates.

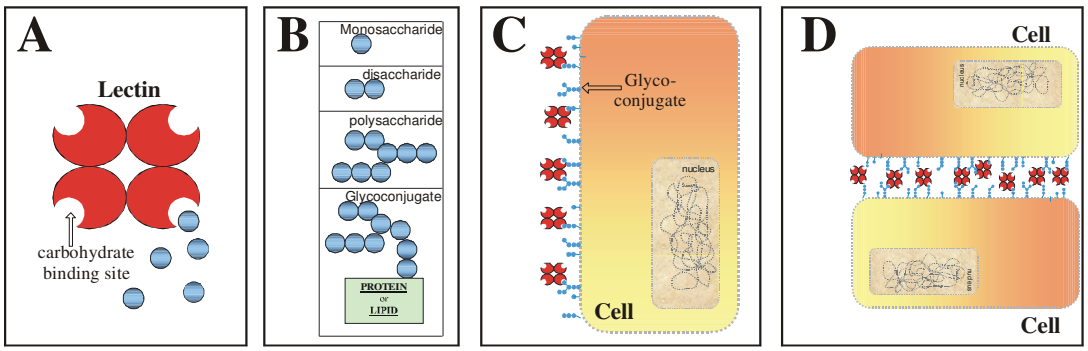

Figure 01: Schematic representation of lectin binding. (**A**) Tetrameric lectin and its four binding sites for carbohydrates. (**B**) Possible binding partners for lectins. (**C**) Lectin binding to glycoconjugates of the cell membrane. (**D**) Cell agglutination caused by lectin binding.

The lectin-induced agglutination of cells has originally served as the most common assay to detect and quantify lectin activity in a variety of organisms (Vlodavsky and Sachs, 1975; Doyle and Keller, 1984; Goldhar, 1995). Despite their broad applicability as a method for the detection of lectin activity, agglutination assays have considerable limitations because only multivalent lectins can agglutinate. Monovalent lectins, with only one binding site for carbohydrates, are usually not detectable by agglutination assays. Therefore, agglutination assays are mostly applied for lectins that are know to have more than one carbohydrate-binding site. Advances in biophysical and molecular biology techniques as well as the availability of synthetic oligosaccharides have contributed to the identification of many lectins. Most lectins have been purified by affinity chromatography (Agrawal and Goldstein, 1967b). For the isolation and characterization of a vast number of lectins, sugar-based polymers like **Sephadex** (glucose), **Sepharose** (galactose) or Chitin (N-acetyl-glucosamine) have been used. Glycoprotein-linked matrices are applied to the purification of lectins that recognize more complex saccharides (Goldstein, 2002). Many lectins have, in addition to the carbohydrate-binding domain, another domain with distinct activity. Proteins that carry lectin domain(s) and other domains with quite different properties have been better studied in animals than in plants (Gabius, 1994).

1. 2. 2 Plant lectins

Lectins are ubiquitous in nature, but the vast majority of isolated and characterized lectins are of plant origin. In plants, lectins are mostly found in storage organs like seeds, but low amounts are also present in leaves, stem, roots or flowers (Rüdiger, 1988; Rüdiger, 1998). The major intracellular location of plant lectins is inside protein bodies, but lectins also occur in the cytoplasm (Pneumans et al., 2000) and in the intercellular space (Etzler et al., 1984). The abundance of plant lectins suggests that they play important roles in plant development and defence against vegetal pathogens and predators (Moreira et al., 1991; Pneumans and van

Damme, 1995; Rüdiger, 1998; Murdock and Shade, 2002). Taking into account where the lectin-binding partners occur, external or internal biological activities are proposed for plant lectins (Rüdiger and Gabius, 2002).

External biological functions of plant lectins serve in the protection against predators like insects, fungi or mammals, or mediate interactions between the lectin-producing plant and another organism. The involvement of plant lectin in interactions with external organisms is best studied in *Leguminosae* plants, which are able to establish a symbiosis with soil bacteria of the genus *Rhisobium* (Brewin and Kardailsky, 1997). The interaction between the plant and bacteria (nodulation) is elicited by the release of bacterial NOD-factors (lipo-chito-oligosaccharides) and by the recognition of these factors by root-lectins (Diaz et al., 1989; van Eijsden et al., 1995). The leguminous lectin from the roots of the plant *Dolichos biflorus* binds to NOD-factors and possesses in addition phosphatase activity (Minic et al., 2000). This lectin is therefore assumed to act as signal transducer (Etzler et al., 1999). Another example of external activity is found in the toxic lectin from *Ricinus communis* (**ricin**) (Stillmark, 1888). Ricin belongs to the "Ribosome-Inactivating Proteins" (RIPs) class of toxins and is a dimeric protein with a lectin subunit (B-chain) and an enzymatic subunit (A-chain) (Pneumans et al., 2001). The lectin subunit of ricin binds to the cell membrane and leads to the penetration of the protein into the animal cell, whereas the enzymatic subunit acts as a specific RNA N-glycosidase on the ribosome (Lord et al., 1994). Ricin depurinates the ribosomal RNA at one specific adenine site. Since the discovery of ricin, a large number of RIPs has been isolated and characterized. They are classified in **type I** (single RNA-glycosidase domain), **type II** (RNA-glycosidase and carbohydrate binding domains) and **type III** (RNA-glycosidase and an unrelated domain with unknown function) (Pneumans et al., 2001). In the past decade, the N-glycosidase activity of RIPs and their substrate specificities have been analyzed in more detail (Barbieri et al., 1996; Barbieri et al., 2000). These studies have shown that some RIPs act as adenosine-glycosidases also on DNA (Ling et al., 1994; Ling et al., 1995; Brigotti at al., 1998; Day et al., 1998; Barbieri et al., 2000; Nicholas et al., 2000). Another example of interaction with DNA is found in the type I RIP from *Iris hollandica* plants, which is able to bind to endogeneous specific genomic DNA-fragments (Hao et al., 2001). It is unlikely that the DNA-binding ability of the mentioned RIPs is related to lectin activity: For two reasons, (i) Type I RIPs have no lectin domain; (ii) type II RIPs, which have their lectin domain removed, are still able to act as adenosine-glycosidase on DNA (Barbieri et al., 1997; Barbieri et al., 2000).

The internal activities and functions of plant lectins are still not completely understood. Since the major location of plant lectins is the protein bodies of the seeds, it has been hypothesized that lectins are just storage proteins (Clarke et al., 1975). The fact that seed lectins

are generally degraded during germination reinforces this hypothesis (van Driesche, 1988). Studies on leguminous lectins during seed germination have shown that the mobilization of these mannose/glucose-specific lectins (tribe *Dioclea*) is always retarded when compared to classical storage proteins (Moreira and Cavada, 1984; Cavada et al., 1990; Cavada et al., 1994). A widely accepted notion of lectin function in seeds is that lectins serve as binding-partners for a variety of storage proteins. This notion has been confirmed by the isolation of seed proteins, called **lectin-binders,** which bind to the lectin from the same plant (Einhoff et al., 1986). Inside the protein body, lectins can also bind enzymes like glucosidases or phosphatases. The phosphatase and the α–mannosidase from *Canavalia ensiformis* seeds interact with **ConcanavalinA** (ConA), the lectin from the same plant (Einhoff and Rüdiger., 1986; Rüdiger and Bartz, 1993). Moreover, the interaction of ConA with α–mannosidase does not involve the carbohydrate binding site of the lectin.

1. 2. 3 The leguminous lectin family

Among plants, the family of *Leguminosae* has received special attention, because the seeds of these plants are particularly rich in lectins (van Driessche, 1988; Kijne et al., 1992; Rougé et al., 1992; Ramos et al., 2000). Most leguminous lectins are protease resistant and survive the passage through the intestinal tract of animals. Therefore, the possible nutritional significance of lectins in leguminous seeds has been extensively investigated (Lajolo and Genovese, 2002). When present in the diet of animals, leguminous lectins have numerous physiological affects, mostly because of their ability to bind to glycoproteins of cells in the small intestine (Putztai et al., 1982; King et al., 1986). Most of the studies on the nutritional effects of lectins derive from animal experiments, where raw legumes or native purified lectins have been used. When purified bean lectins are used to feed rats, a decrease in rat growth, an enlargement of the small intestine, damage of the epithelium of the small intestine and hypertrophy of the pancreas has been observed (King et al., 1986). Some of these effects are due to lectin-related increases in the expression of transforming growth factors as well to decreases in the levels of heat shock proteins (Putztai et al., 1990; Putztai, 1993; Dignass et al., 1996; Ovelgonne et al., 2000). Studies on the lectin from common beans (*Phaseolus vulgaris* agglutinin - PHA) in rats have also revealed that this lectin binds to the gastric mucosal and parietal cells and inhibits gastric acid secretion (Kordas et al., 2000; Kordas et al., 2001). These effects of leguminous lectins have raised questions about the consequences of legume consumption by humans (Lajolo and Genovese, 2002). But the significance of the dietary effects of leguminous lectins in humans is considered low, because legumes are normally consumed after previous heat treatment that inactivates lectin activity (Messina, 1999).

1. 2. 4 Properties of leguminous lectins

A large number of lectins from the *Leguminosae* family have been isolated and characterized (Sharon and Lis, 1990). They resemble each other in their physicochemical properties and structures, but exhibit different carbohydrate specificities (Reeke and Becker, 1988). The carbohydrate binding specificity of some legume lectins are presented in Appendix (Table 01 and 02). Leguminous lectins usually consist of two or four subunits (25-30 kDa). Each subunit or monomer contains one sugar binding site and two binding sites for metal ions (Ca^{2+} and Mn). Comparisons of the amino acid sequences among leguminous lectins indicate that these proteins have been well conserved during evolution (Van Driesche, 1988; Rougé et al., 1992). The primary structures of the leguminous lectins are remarkably homologous, with approximately 20% of invariant amino acids and almost 20% of similar ones. The conserved amino acids include several that participate in hydrogen bonding or in hydrophobic interactions with the carbohydrate (Sharon and Lis, 2002). Almost all amino acids that coordinate the metal ions are conserved.

1. 2. 5 Structural aspects of leguminous lectins

The structure of monomers of the leguminous lectins resembles that of a flattened bell-shaped dome. The structure of the monomer presents seven (front face) and six (back face) antiparallel strands of β-sheets interconnected by turns and loops (Fig. 02). The antiparallel strands of β-sheets present the topology of the **jelly roll fold** known as "the lectin fold"(Srinivasan et al., 1996). The same fold has been found in other non-plant proteins including the mammalian galectins. The structure of the dimer results normally from the side-by-side association of two subunits. The tetrameric association of leguminous lectins involves side-by-side as well as back-to-back interfaces (Fig. 02). The disposition of the back β-sheets to one another defines the overall quaternary arrangement (Prabu et al., 1999) (Fig. 02 B). The tetramers of ConA, SBA and PHA-L can be seen as made up of the back-to-back association of two dimers (Mitra et al., 2002). Many leguminous and animal lectins share similarities in folds and in the architectures of the carbohydrate binding site (Loris et al., 2002) (Fig. 02 C).

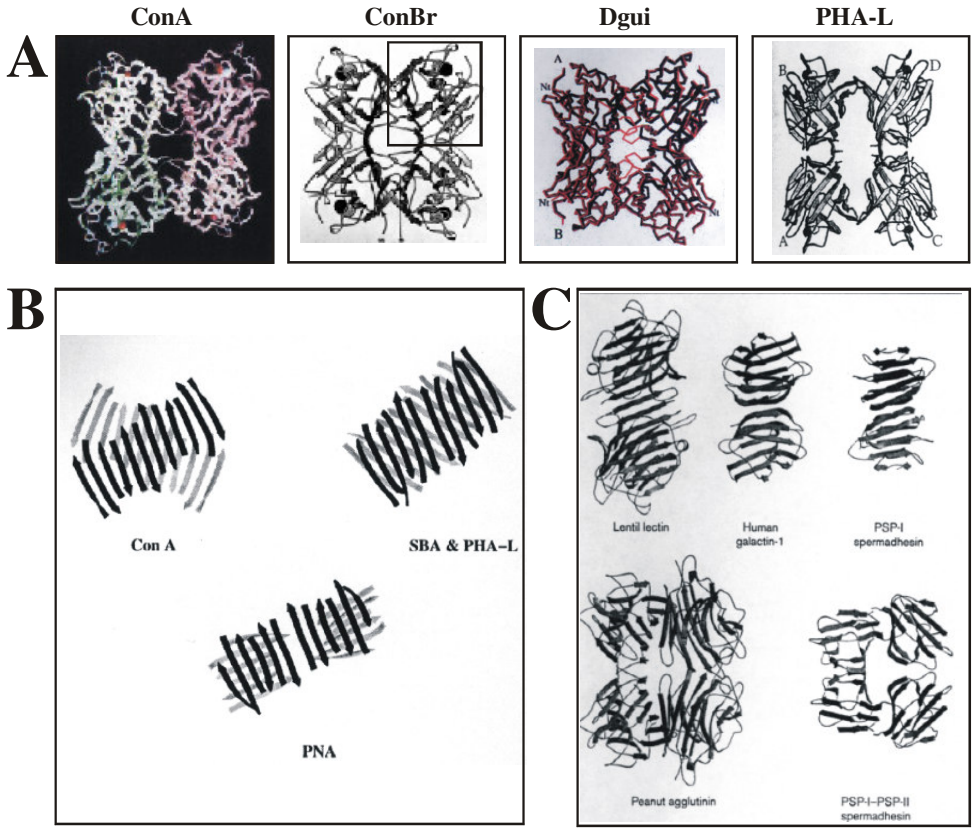

Figure 02: **Structural organization of legume lectins.** (**A**) Tetrameric structures of lectins from *Canavalia ensiformis* (ConA), *Canavalia brasiliensis* (ConBr), *Dioclea guianensis* (Dgui) and *Phaseolus vulgaris* (PHA-L) (Hamelryck et al., 1996; Naismith and Field, 1996; Sanz-Aparicio et al., 1997; Wah et al., 2001). The **β-sheet jelly roll** organization of the subunit is outlined in the ConBr-tetramer. (**B**) Pattern of quaternary association in legume lectins showing the relative positions of the dimers in the formation of the each tetramer (Pabru et al., 1999). (**C**) Comparisons between the structures of leguminous and animal lectins (Bouckaert et al., 1999).

1. 2. 6 Concanavalin A and ConA-like lectins

The best-studied plant lectin, *ConcanavalinA* (ConA), has been isolated from the seeds of *Canavalia ensiformis*, which belongs to the Leguminosae family (Sumner, 1919; Sumner and Howell, 1936; Agrawal and Goldstein, 1967a; Edelman et al., 1972;). ConA binds specifically to glucose or mannose and has the ability to precipitate complex polysaccharides as glycogens, mannans and dextrans (Agrawal and Goldstein, 1967b; Hardman and Ainsworth, 1976). Since glucose and mannose are frequently found in the membrane glycoconjugates of a great number of cells, ConA can bind to many different cell types from a broad range of organisms (Barat and Avrameas, 1973; Rottmann et al., 1974; Skehan and Friedman, 1975; Maca, 1976; Furmanski et al., 1981; Storrie and Maurey, 1981; Zhao et al., 2002). *Canavalia ensiformis* belongs to the *Diocleinae* subtribe. Lectin members of this subtribe share many characteristics. They are all mannose/glucose specific and have similar quaternary structures. Most *Diocleinae* lectins show

high affinity for the core trimannoside of asparagine-linked oligosaccharides (Dam et al., 1998). Because of their homologies to ConA, many *Diocleinae* lectins are called ConA-like.

The dimer/tetramer association of some legume lectins is dependent on pH. Particularly in the case of ConA, the equilibrium between the dimeric and tetrameric conformations is highly pH-dependent (McKenzie and Sawyer, 1973; Senear and Teller, 1981a). ConA exists as dimer at pH values below 5.0, as tetramer at pH over 7.0 and as a mixture of dimers and tetramers at pH values between 5.0 and 7.0 (Senear and Teller, 1981b).

Each monomer of ConA has one saccharide binding site, a transition metal ion (generally Mn^{2+}) binding site S1, and a Ca^{2+} binding site S2 (Becker et al., 1975). ConA can exist as a mixture of two conformational states which has been denominated as the "**locked**" and the "**unlocked**" forms (Brown et al., 1977; Sherry et al., 1978; Hardman et al., 1982; Brewer et al., 1983a; Bouckaert et al., 2000). The locked form binds two metal ions per monomer and has high affinity for saccharide (Karlstam, 1973; Stark and Sherry, 1979). The unlocked form binds weakly to metal ions and saccharides, it is the conformation more frequently found of metal-free ConA (Shoram et al., 1978; Brown et al., 1982; Brewer et al, 1983b; Bouckaert et al., 1995).

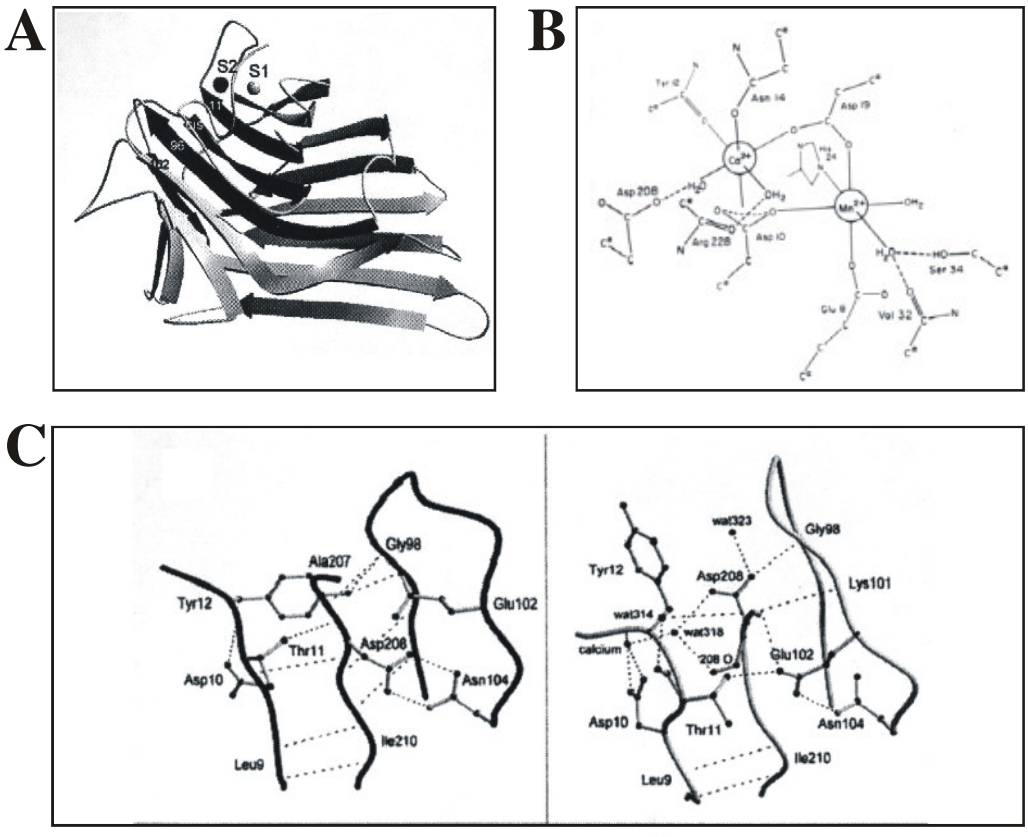

Figure 03: 3D-Structure, metal binding and conformations of the ConA-subunit. (**A**) Three-dimensional structure of the ConA monomer and the relative location of S1 and S2 metal binding sites (Bouckaert et al., 1995). (**B**). Schematic representation of the binding of metals to ConA (Becker et al., 1975). (**C**) Structural differences between the "unlocked" (left) and the "locked" (right) (Bouckaert et al., 1995).

Additionally to its mannose/glucose-binding activity, ConA can bind to glycosaminoglycans by ionic interactions (Toda et al., 1981). The binding occurs only at low ionic strength and pH. Hydrophobic interactions of ConA have also been reported (Ochoa et al., 1979). This non-specific interaction is enhanced by the addition of salt and is independent of the sugar-binding site.

1. 2. 7 Animal lectins

In the past decades, an increasing number of human, animal, bacterial, viral and fungal lectins have been isolated and characterized (Sharon, 1987; Ward et al., 1990; Gabius, 1991; Vasta et al., 1994; Petri et al., 2002). Since the discovery of lectins occurring in many different organisms, the biological functions of these proteins have been a matter of discussion. Attempts to find a general role for all lectins have failed, probably because the functions of each of these proteins depend on the organism considered. Animal lectins are thought to be involved in a variety of important cellular functions, such as cell recognition, tissue and organ formation, innate immune defense, tumor metastasis, fertilization, migration and recruitment of lymphocytes (Gabius, 1987; Kieda, 1998; Angata et al., 2002; Cebo et al., 2002). In addition to the fact that a single organism can express more than one type of lectin, the location and the time of their appearance account for the complexity of their functions. Although lectins vary in sizes and shapes, they can be grouped into distinct families with similar amino acid sequence and structure (Sharon and Lis, 1990; Loris et al., 1998; Rini and Lobsanov et al., 1999).

The functional diversity and the structural characteristics of animal lectins allow their classification into discrete lectin families. The carbohydrate-binding activity of many animal lectins are due to the presence of a conserved polypeptide segment designated the Carbohydrate-Recognition Domain (**CRD**). Major mammalian lectin families are the **galectins**, Ca^{2+}-binding lectins (**C-type),** mannose-6-Phosphate-binding lectins (**P-type**) and immunoglobulin-like lectins (**I-type**) (Drickamer, 1995). Galectins are soluble lectins with high affinity for β-galactoside-containing sugars (Kasai and Hirabayashi, 1996; Brewer, 2002; Rabinovich et al., 2002a). There are more than 13 mammalian galectins characterized, which are involved in cell growth, proliferation, apoptosis, cell-matrix-interactions or in the inflammatory response (Lobsanov, et al., 1993; Hernandez and Baum, 2002; Rabinovich et al., 2002b). C-type lectins bind sugars through CDR-domains in a calcium-dependent manner and are involved in immunity modulation (Figdor et al., 2002; Engering et al., 2002). C-type lectins are either produced as transmembrane proteins or secreted as soluble proteins. A group of C-type lectins, called **collectins**, includes the lung surfactant proteins (SP-A and SP-D), which are secreted at the luminal surface of pulmonary endothelial cells, and the mannose-binding protein (MBP), which

is present in plasma. Collectins are involved in innate immunity (Hoppe and Reid, 1994; Hakasson et al., 2000; McCormack and Whitsett, 2002). Transmembrane C-type lectins include the E, L and P-**selectins**, which play a role in leukocyte trafficking and migration (Sharon and Lis, 1995; Zollner and Asadullah, 2003), and the mannose receptor family which is involved in the binding and uptake of pathogens (East and Isacke, 2002; Cambi and Fidgor, 2003). A variety of transmembrane lectins and lectin-like receptors are considered to act in signal transduction (Hebert, 2000).

Important aspects of animal lectin functions are intrinsically related to their cellular locations. Some intracellular animal lectins function as sorting receptors and chaperones in the secretory pathways (Hauri et al., 2000; Storrie and Nilson, 2002; Schrag et al., 2003). The endoplasmic reticulum specific **calnexin** and **calreticulin**, which bind to the terminal glucose of high mannose N-linked glycans, fall into this group (Ware et al., 1995; Hebert et al., 1995; Spiro et al., 1996; Kapoor et al., 2002). The mannose-specific lectins found in the ER-Golgi intermediate compartment (ERGIC-53) and in the Golgi complex (VIP-36) share structural similarities to legume lectins (Fiedler and Simons, 1994; Itin et al., 1996; Velloso et al., 2002). Moreover, the lectin domain of calnexin is structurally similar to the ConA-like lectins (Murzin et al., 1995; Lo Conte et al., 2002).

1. 2. 8 Applications of lectins in gene transfer

Like other members of the leguminous lectin family, ConA can survive proteolysis during its passage through the digestive tract of animals and recognize the carbohydrate moieties of intestinal cells (Pusztai et al., 1990). For that reason, leguminous lectins are considered potentially attractive tools for the lectin-mediated mucosal delivery of drugs (Clark, 2000; Woodley et al., 2000). As the oral route and the gastrointestinal tract are the most frequent portals of entry for therapeutics in humans, lectins have become potential tools for gene therapy (Erbacher et al, 1995; Monsigny et al., 1999; Yamazaki et al., 2000; Roche et al., 2003). DNA has been shown to interact with many different proteins either non-specifically or in a highly sequence-specific manner. Our laboratory has had a long-standing interest in the interaction of DNA with mammalian cells and organisms (Groneberg et al., 1975; Wienhues et al., 1986; Schubbert et al., 1997). The knowledge that leguminous lectins are able to bind to the membranes of many eukaryotic cells has stimulated our interest in the application of lectins to the uptake of DNA by mammalian cells.

1. 3 Aim of this Investigation

The notion that the functions of lectins are restricted to their carbohydrate binding abilities has been challenged by the discovery of an increasing number of lectins with additional binding sites for non-sugar ligands. The properties of lectins that possess such additional binding abilities have been better characterized in animal than in plants lectins. The majority of the plant lectins isolated and characterized belong to the *Leguminosae* family. The variability of their carbohydrate specificities, biological activities and availability has contributed for their wide application in many areas of biological research. Nevertheless, the use of leguminous lectins is still primarily based on their carbohydrate binding activities. The identification and characterization of additional binding sites in leguminous lectins is relatively limited. Investigations directed toward the identification of new binding abilities of leguminous lectins would, therefore, contribute to the characterization these proteins.

The main purpose of the investigation presented here has been the identification and characterization of the ability of leguminous lectins to bind to DNA. For that purpose, a number of leguminous lectins have been studied for their ability to bind to DNA in solution and to form stable lectin-DNA-complexes. I have tried to answer following questions:

(1) Are leguminous lectins able to bind to DNA?
(2) What are the experimental conditions that allow lectin-DNA binding?
(3) Is the carbohydrate binding site of the lectin involved in DNA-recognition?
(4) What are the properties of lectin-DNA-complexes?

2. MATERIAL

2. 1 Reagents

Acetic acid	Merck, Darmstadt
Agarose (″ultra pure″)	Gibco, Eggenstein
Bovine serum albumin (BSA)	New England Biolabs, Beverly, MA, USA
Bromphenol blue	Merck, Darmstadt
Chloroform	J. T. Baker Chemicals, Deventer Niederlande
Coomassie Blue R-250	Merck, Darmstadt
Dextran sulfate, Natrium	Biomol Fine Chemicals, Hamburg
D, L – dithiothreitol (DTT)	Sigma Chemicals, St. Louis, MO, USA
DNA marker (for agarose electrophoresis)	Fermentas MBI, Vilnius, Litauen
Dodecylsulfate, Natrium (SDS)	Merck, Darmstadt
Ethanol	Merck, Darmstadt
Ethidium bromide	Calbiochem, La Jolla, CA, USA
Ethylendiamintetraacetate (EDTA)	Merck, Darmstadt

Fetal calf serum (FCS)	Cytogen, Berlin
Glycerin (87%, ultra pure)	J. T. Baker Chemicals, Deventer Niederlande
Hexa-oligo-deoxyribonucleotide (ramdom primer $p(dN)_6$)	Boehringer Mannheim, Mannheim
8-hydroxychinoline	Merck, Darmstadt
Magnesium chloride	Merck, Darmstadt
Milk powder (Glücksklee Magermilchpulver)	Nestlé, Frankfurt am Main
Natrium acetate	Merck, Darmstadt
Natrium chloride	Merck, Darmstadt
Natrium citrate	Merck, Darmstadt
Natrium hydrogen phosphate	Merck, Darmstadt
Natrium hydroxide	Roth, Karlsruhe
Phenol	J. T. Baker Chemicals, Deventer, Niederlande
Salmon sperm DNA	Sigma, Chemicals, St. Louis, MO, USA
Salzsäure (rauchend)	Merck, Darmstadt
5‘-triphosphate-deoxyribonucleosides	Promega, Madison, WI, USA
Tris (2-Amino-2-hydroxyl-methyl-propan-1,3-diol)	Merck, Darmstadt

Xylene cyanol	Fluka, Buchs SG, Basel, Schweiz

2. 2 Other Laboratory Materials

Serings	Braun, Melsungen
Needles	Terumo, Leuven, Belgien
Develop machine (Curix 60)	Agfa-Gavaert, Mortsel, Belgien
Cassete for Films	Suprema Filmkassetten (Dr. Goos Universal)
Cell culture flasks ($25\ cm^2$, $75\ cm^2$, und $175\ cm^2$)	Becton Dickinson (Falcon), Oxnard, CA, USA
Cell culture flasks (for CO_2 incubator) ($25\ cm^2$, $75\ cm^2$, und $185\ cm^2$)	Nunc
Cell culture plates (6-, 24 and 96 wells)	Becton Dickinson (Falcon), Oxnard, CA, USA
Cell counter slide (Neubauer)	Brand, Wertheim
Centrifuges	Beckman, Sorvall, Heraeus, Eppendorf
Centrifuge tubes, Ultra-Clear (SW 60)	Beckman Instruments Inc., Palo Alto,CA, USA
Cintillation counter (LS 1801)	Beckman, Palo Alto, CA, USA

Cryotubes (1,8 ml)	Nunc, Wiesbaden
Dialysis bags	Medicel International, London, UK
Eppendorf tubes (1,5 ml)	Eppendorf, Hamburg
Falcon tubes, 50 ml	Falkon, Becton Dickinson, Lincoln Park, USA
Falcon tubes, 15 ml	Falkon, Becton Dickinson, Lincoln Park, USA
Film Kodak EPL 400	Kodak, Stuttgart
Film Kodak (X-OMAT^{TM} AR)	Eastman Kodak Company, Rocherster, NY, USA
Fraction collector	Pharmacia Biotech, Uppsala, Schweden
Glas cover slides	Menzel, Fleischhacker
Glas reaction tubes	Schott, Mainz
Hybridisierungsinkubator	Backofer, Reutlingen
Hybridisation incubators (68 °C)	Backofer, Reutlingen
Incubators (37 °C)	Heraeus Sepatech, Hanau
Kimwipes	Kimberly Clark, EEC
Liquid Nitrogen tanks	L'air Liquide, Champigny-sur-Marne, Frankreich
Microscope (Leitz)	DiavertLeitz, Wetzlar

Microscope (Zeiss)	Karl Zeiss, Jena
Microscope slides	Menzel-Gläser, Mainz
Nylon plus membrane (positive charged)	Qiagen, Hilden
Peristaltic pump	Pharmacia Biotech, Uppsala, Schweden
pH-Indicator	Merck, Darmstadt
Pipetes (0,5 – 10 µl und 100 – 1000 µl)	Eppendorf, Hamburg
Pipetes (1 – 10 µl, 1 – 20 µl und 100 - 1000µl)	Gilson, medical eletronics, Villers-le-Bel
Pipete pump pipump	Glasfirm, Gießen
Pipete pump Fortuna Labopet 240	Greiner, Frickenhausen
Pipete tips	Eppendorf, Hamburg
Pipete tips	SLG, Gauting
Plastic bags (for Southern-membranes)	Bio Rad
Plastic sheets	Melitta, München
Quarz kuvete (Suprasil)	Beckman, Palo Alto, CA. USA
Reaction tubes (1,5 ml und 2 ml)	Brand, Wertheim Eppendorf, Hamburg
Reaction tubes (white cap, 10 ml)	Greiner, Frickenhausen

Sterile bench (Bio Gard Hood)	Baker Company, Sanford, CT, USA
Spektrophotometer (DU 640)	Beckman, Palo Alto, CA. USA
Sterile becht (Antair BSK 4)	Anthos Mikrosysteme, Köln
Sterile filters (0,2 µm und 0,45 µm Porendurchmesser)	Schleicher & Shuell, Dassel
Ultracentrifuge (L7 55, L8 55m und L8 70)	Beckman , Palo Alto,CA, USA
Video documentation system (CS 1)	Cybertech, San Gabriel, CA, USA
Vortex Genie 2	Bender & Hobein, Zürich, Schweiz
„Whatman“-paper	Schleicher & Shuell

2. 3 Radioisotopes

Deoxyribonucleosid-5‘-[α-^{32}P]-triphosphate (specific activity > 3000 Ci/mmmol)	New England Nuclear (NEN), Du Pont, Boston, MA, USA

2. 4 Enzymes

2. 4. 1 Restriction Endonucleases

The restriction endonucleases HaeII, EcoRI and PstI were purchased from MBI Fermentas (Vilnius, Litauen) and the incubation conditions used were as recommended by the supplier.

2. 4. 2 Other Enzymes

DNA-Polymerase I (Klenow-Fragment)	Boehringer Mannheim, Mannheim
Proteinase K	Merck, Darmstadt
RNase A	Sigma, St. Louis, MO, USA
Trypsin 1-300	United States Biochemical Corporation, Cleveland, OH, USA

2. 5 Lectins

ConA (*Canavalia ensiformis*), PHA-E (*Phaseolus vulgaris* erythroagglutinin), PHA-L (*Phaseolus vulgaris* leucoagglutinin) and VVLB4 (*Vicia villosa* isolectin B4) were purchased from Sigma Aldrich (St. Louis, Mo). LcA (*Lens culinaris* agglutinin), SBA (Soybean agglutinin) and PNA (Peanut agglutinin) from Calbiochem (Bad Soden). The ConBr (*Canavalia brasiliensis*), ConBn (*Canavalia bonariensis*), ConG (*Canavalia grandiflora*), ConM (*Canavalia maritima*), Dviol (*Dioclea violacea*) and Dvirg (*Dioclea virgata*) lectins were prepared as described elsewhere (Moreira and Cavada, 1984; Cavada et al., 1990; Moreira et al., 1991; Cavada et al., 1994; Dam et al., 1998; Ramos et al., 2000). The purity of all lectins was checked by SDS-polyacrylamide gel electrophoresis.

2. 6 DNA

The plasmid pEGFP-C1 carried the gene for the green fluorescent protein (GFP) from *Aequorea victoria* in the pUC19 plasmid under the control of the SV40 early promotor. The plasmid purchased from Clontech (see Appendix, Fig 36) was produced in large scale using the CsCl gradient ultracentrifugation standard methods. The isolated plasmid preparation contained predominantly closed supercoiled circular plasmid and, in lower amounts, nicked and oligomeric plasmid forms. Restriction analysis of the purified plasmid DNA was performed to confirm the identity of the fragment sizes compared to the pEGFP-C1 restriction map. The Lambda-DNA

(*Eco*130I/*Mlu*I) size marker as well as the 100 bp DNA-marker used in control experiments to analyze the ConA-DNA complexes for possible sequence specificities were purchased from MBI fermentas (Vileins, Lithuania).

2. 7 Cells

2. 7. 1 Mammalian Cell Lines

The cell lines used for agglutination assay as well as for the pEGFP-C1 DNA uptake and GFP expression were obtained from ATCC (American Type Culture Collection). The cells were grown in Dulbecco modified medium supplied with 10 % FCS.

A 549 (CCL 185	Human Lung Carcinom
BHK21 (CCL 10)	Baby Hamster Kidney Cells
CHO-K1 (CCL 61)	Chinese Hamster Ovary Cells
CaCo-2	Human Enterocytes

2. 7. 2 Human Erythrocytes

The human erythrocytes of blood group O were donated by the Zentrum für Transfusionsmedizin (University of Cologne, Germany) and were storaged at 4 °C until use.

2. 8 Chromatographic Matrices

Bio-Gel A agarose (15m fine)	Bio Rad
Sephadex G25 (NAP-10™)	Pharmacia Biotech, Uppsala, Schweden

Sephadex G75	Pharmacia Biotech, Uppsala, Schweden

2. 9 Solutions, Buffers and Culture Media

Chloroform	Chloroform und Isoamylalkohol at 24 : 1 ratio
Coomassie solution (for SDS-PAGE)	50% methanol 10% acetic acid 0,25% Coomassie Blue R-250
Cryo-medium (for cryopreservation of cells)	10 % FCS 10 % Glycerin 80 % Dulbecco Medium
CsCl solution (for the gradient ultracentrifugation)	0,5 g/ml in 20mM Tris-HCl, pH 7,5 (refraction index 1,369)
Denaturation solution (for Southern Blot)	0,4 M NaOH
Depurinisation solution (for Southern Blot)	0,25 M HCl
Destain solution (for SDS-PAGE)	40% methanol 7% acetic acid
Deoxyribonucleoside-5‘-triphosphate	10 mM in dH_2O
Dextransulfate stock solution	50% in dH_2O
DTT (Dithiothreitol)	1,0 M in dH_2O (steril filtrated)

Dulbecco Modifield Medium	Bablanian et al., 1965
EDTA	0,5 M in dH_2O, pH 8,0
Ethidium bromide solution	2 µg /ml in dH_2O
Hybridisation solution (for Southern Blot)	2 x SSC 1 % SDS 0,5 % Milk powder 0,5 mg/ml Salmon Sperm DNA 10 % Dextran sulfate
Milk powder mix (10 X)	5 % Milk powder 10 % SDS
$MgCl_2$ solution	1 M in dH_2O
Natrium acetate solution (for DNA precipitation)	3 M Natrium acetate pH 5,2 (adjusted with acetic acid)
Oligo Labeling Buffer (5 x OLB)	50 mM Tris-HCl, pH 8,0 5 mM $MgCl_2$ 5 mM DTT 200 mM HEPES, pH 6,6 250 g/ml ramdom primer (dN_6)
PBS (Dulbecco und Vogt, 1954)	140 mM NaCl 3 mM KCl 8 mM Na_2HPO_4 1 mM KH_2PO_4 1 mM $MgCl_2$ 1 mM $CaCl_2$ 0,03 % Penicillin 0,015 % Streptomycin

PBS-d	PBS without $MgCl_2$, $CaCl_2$ and antibiotics
Phenol	saturated with 0,1 M Tris-HCl, pH 7,5
Prehybridisation solution (for Southern-Blot)	2 X SSC 1 % SDS 0,5 % Milk powder 0,5 mg/ml Salmon sperm DNA
Proteinase K stock solution	10 mg/ml in 10 mM Tris-HCl, pH 7,5
RNase A (stock solution)	10 mg/ml in dH_2O (the solution is boiled for 15 min, equilibrated to room temperature, alyquoted and storaged at –20°C.)
Salmon Sperm DNA	10 mg/ml in dH_2O
SDS stock solution	10% in dH_2O
SSC (1 X) (Standard Saline Citrate)	150 mM NaCl 15 mM Na-Citrat (pH adjusted to 7,0 with citric acid)
Stop buffer	100 mM EDTA, pH 8,0 1 % SDS 0,1 % Bromphenol blue 0,1 % Xylenxyanol 70 % Glycerin
TE-buffer	10 mM Tris-HCl, pH 7,5 1 mM EDTA, pH 8,0
Tris 1M (for SDS-PAGE)	1 M Tris in dH_2O (pH adjusted to 6,8 with cloridic acid)

Tris-HCl (Stammlösung)	1 M Tris in dH_2O (pH adjusted to 7,5 with cloridic acid)
Tris saline	14 mM NaCl 5 mM KCl 0,3 mM Na_2HPO_4 25 mM Tris-HCl, pH 7,4 0,1 % Glucose (w/v) 0,03 % Penicillin (w/v) 0,02 % Streptomycin (w/v)
Trypsin solution	0,25% in Tris saline
Wash buffer I (for Southern-Blot)	2 X SSC 0,1 % SDS
Wash buffer II (for Southern-Blot)	0,1 X SSC 0,1 % SDS

3. METHODS

3. 1 Lectin-DNA binding reaction

3. 1. 1 Lectin binding reaction with closed pEGFP-C1 plasmid DNA

Lyophilized lectin powder was solubilized in 10 mM Tris-HCl pH 7.5 (binding buffer) to yield a 10 µg/µl stock solution. The concentration of the stock solution was confirmed by measuring the absorbance at 280 nm wavelengths, taking the specific Extinction Coefficient of the lectin as reference (Agrawal and Goldstein, 1967a). For each new experiment a fresh stock solution was prepared.

DNA stock solutions of the pEGFP-C1 in TE-buffer (10 mM Tris pH 7.5, 1 mM EDTA) were maintained at 4°C and appropriate DNA solutions were prepared just before each experiment. Typically, the DNA solutions were prepared in binding buffer at 60 ng/µl and stored on ice until used.

Serial dilutions of the lectins were prepared from lectin stock solutions in binding buffer. Binding reactions were performed in a total volume of 25 µl by adding DNA at 6 ng/µl to lectin samples at different concentrations. While the DNA concentration in the lectin–DNA mixtures was kept constant, the lectin concentrations were 9, 6.75, 5.063 , 3.797, 2.848, 2.135, 1.602, 1.201, 0.901, 0.676, 0.506, 0.380, 0.285, 0.214 and 0.160 µg/µl in different experiments. As negative control, the DNA was mixed with binding buffer devoid of lectins. After the lectin-DNA mixtures or the negative control were incubated at 37°C for 30 min, 4 µl of loading buffer (100 mM EDTA, bromophenol blue / xylenocyol / glycerol) was added, and samples were loaded on agarose gels for electrophoresis.

3. 1. 2 Lectin binding reactions with linearized pEGFP-C1 plasmid DNA

Linearized pEGFP-C1 plasmid DNA was prepared by treatment with either EcoRI or PstI restriction enzyme. The restriction reaction was performed by using 10 units of enzyme per µg DNA for 1 h at 37°C. The linearized DNA was EtOH-precipitated and, after centrifugation, the pellet was resuspended in TE-buffer. A DNA working solution of the linearized pEGFP-C1 plasmid at 60 ng/µl was prepared in binding buffer just before use. Lectin serial dilutions as well as the lectin-DNA binding reaction were performed as described under 3. 1. 1.

3. 1. 3 Lectin binding reactions with pEGFP-subfragments and DNA-fragments from different sources

To investigate the possibility of sequence specificities in the binding of lectins to DNA, the reaction was performed with pEGFP-C1 subfragments as well as with DNA from different sources and of different sizes (pUC Mix and 100bp DNA markers). The pEGFP-C1 DNA subfragments were obtained by cleavage of the plasmid with MspI or HaeII enzyme. Restriction reactions were performed using 10 units of enzyme per µg DNA for 1 h at 37°C. DNA fragments were EtOH-precipitated and, after centrifugation, pellets were resuspended in TE-buffer. Commercially available DNA size markers (pUC Mix Marker and GeneRuler^{TM} 100bp DNA Ladder) were solubilized in TE-buffer, and the appropriate DNA-working solutions were prepared in binding buffer at 167 ng/µl just before use.

Lyophilized lectin powder was solubilized in 10 mM Tris-HCl pH 7.5 (binding buffer) to yield a 10 µg/µl stock solution. Serial dilutions of the lectin were prepared from the stock solution in binding buffer. Binding reactions were performed in a total volume of 30 µl by adding DNA at 16,7 ng/µl to lectin samples at different concentrations. Lectin serial dilutions, binding reaction incubation conditions, as well as electrophoresis analysis were performed as described under 3. 1. 1.

3. 2 Detection of lectin-DNA-complexes

3. 2. 1 Electrophoresis of the lectin-DNA-complexes

Reaction mixtures were electrophoretic separated overnight at 40 V in 0.8% agarose gels (20x25 cm) in 0.5 X TBE buffer, which consist of 90 mM Tris, 90 mM boric acid, 2.5 mM-EDTA. After staining the DNA with ethidium bromide, 0.5 µg/ml, gels were UV photographed.

3. 2. 2 Southern transfer of the lectin-DNA-complexes to positively charged nylon membranes

For the transfer of the lectin-complexed DNA from electrophoretic gels to positively charged nylon membranes, gels were incubated in 0.25 M HCl for 20 min, promoting partial DNA depurination. Subsequently, gels were incubated in 0.4M NaCl to denaturate the DNA. Finally, the DNA was blotted over night to positively charged nylon membranes (GeneScreen

Plus, NEN®) using the procedure described elsewhere (Southern, 1975; Koetsier et al, 1993).

3. 2. 3 Radioactive labeling of the DNA-probes and hybridization of the DNA on nylon membranes

The pEGFP-C1 plasmid was ^{32}P-labeled by random priming and used as hybridization probe. Standard protocols were used for hybridization, washing of membranes and autoradiography on Kodak X-OMAT films (Southern, 1975; Koetsier et al, 1993).

3. 3 Competition experiments

3. 3. 1 Binding in the presence of 2-D-deoxyribose

In an attempt to find out the binding site of ConA on the DNA molecule, constant amounts of this lectin were incubated with variable amounts of 2-D-deoxyribose prior to the addition of the DNA-solution. Serial dilutions of 2-D-deoxyribose and a ConA stock solution at 10 µg/µl were prepared in TE-buffer. A reaction volume of 27 µl contained 23.4 µl of each 2-D-deoxyribose dilution and 3.6 µl of the ConA stock solution. After an incubation of 30 min at 37°C, 3 µl of pEGFP-C1-DNA solution was added to each sample, followed by incubation at 37°C for another 30 min. The total reaction volume of 30 µl contained 6 ng DNA per µl, 1.2 µg of lectin per µl and a final concentration of 2-D-deoxyribose between 100 mM and 3 µM. Positive controls contained no sugar and negative controls no lectin, but 2-D-deoxyribose at a final concentration of 100 mM. Sugar was added to the negative control to test for possible effects of this component on the electrophoretic mobility of DNA. The DNA binding ability of the lectin under the described conditions was analyzed by electrophoresis and Southern-blot hybridization.

3. 3. 2 Binding in the presence of specific sugars

The competition for DNA-lectin binding by specific sugars was assessed by preincubating ConA for 30 min at 37 °C with different sugars. Subsequently, the DNA was added, followed by incubation at 37 °C for 30 min. Solutions of the sugars α-methyl-D-glucopyranoside, α-methyl-D-mannopyranoside or galactose at concentrations between 1 M and 31.25 mM in TE-buffer were tested for their effects on complex formation at constant lectin and DNA concentrations. Typically, a total reaction volume of 40 µl contained 6 ng DNA per µl, 1.2

µg of lectin per µl and a final concentration of sugar between 200 mM and 6.25 mM. Positive controls contained no sugar and negative controls no lectin, but α-methyl-D-glucopyranoside at a final concentration of 200 mM. Sugar was added to the negative control to test for a possible influence of this component on the electrophoretic mobility of DNA. The DNA binding ability of the lectin was then analyzed by electrophoresis and Southern-blot hybridization.

3. 3. 3 Binding in the presence of ATP

The competition for DNA-lectin binding by ATP was assessed by preincubating ConA with ATP for 30 min at 37 °C. Subsequently, the DNA was added, followed by incubation at 37 °C for 30 min. ATP solutions at concentrations between 60 mM and 0.4685 mM in TE-buffer were tested for their effects on complex formation at constant lectin and DNA concentrations. Typically, a total reaction volume of 40 µl contained 6 ng DNA per µl, 1.2 µg of lectin per µl and final concentration of ATP between 10 mM and 78.12 µM. Positive control contained no a ATP and negative control no lectin. After incubation, samples were analyzed by electrophoresis and Southern-blot hybridization.

3. 3. 4 Binding in the presence of anti-ConA-antibody

Since the location of a DNA recognition binding site on ConA was not known, it was investigated if the occupation of multiple positions in ConA-molecules by polyclonal anti-ConA-antibody would decrease or inhibit ConA-DNA-complex formation. In this experiment, ConA was first exposed to the antibody and then DNA was added to the mixture. The resulting mixtures were finally analyzed by electrophoresis and Southern blot hybridization.

Anti-ConA-antibody (VECTOR laboratories) stock solutions at 2 µg/µl were prepared in binding buffer, aliquoted and stored for long term at –20°C. Before each experiment, an aliquot was thawn on ice. Serial dilutions of the antibody were prepared from the stock solution in binding buffer. ConA solution was added to each antibody dilution and the mixtures incubated at 37°C for 30 min. After incubation, pEGFP-C1-DNA solution was added, and samples were reincubated for another 30 min at 37°C. In a final volume of 180 µl, each sample had ConA at 1.2 µg/µl and DNA at 6 ng/µl. The antibody concentrations were respectively 278, 139, 69.5, 34.75, 17.38, 8.69, 4.34, 2.17, 1.09 and 0.54 ng/µl. Positive control contained no antibody and negative control no ConA. Electrophoretic analysis was performed using 50 µl of each sample and 7µl of loading buffer.

3. 4 Kinetics of the ConA-DNA-complex formation

To estimate the kinetics of formation of ConA-DNA-complexes in solution, a constant amount of ConA was incubated with a constant amount of DNA for a variety of time intervals. In a total volume of 40 µl, ConA at 1.2 µg/µl was incubated with DNA at 0.006 µg/µl. ConA-DNA-complexes were allow to form at 37 °C for different times of incubation, and directly applied to a 0.8% agarose gel for electrophoresis. The negative control contained no ConA.

3. 5 Influence of DNA concentration on the ConA-DNA-complex formation

The characterization of ConA-DNA-complexes was mostly carried out at variable ConA-concentration and constant DNA-amount. An experiment designed to maintain ConA-concentration constant and to vary the DNA amount had shown to be a more complicated task, because the DNA stained on gels and hybridized in Southern-Blot would vary too much from sample to sample. To test the influence of DNA concentration on the ConA-DNA-complex formation it was necessary to perform the binding reactions in big volumes and apply in the electrophoretic gel just the volume necessary to have an equal amount of DNA per sample. The binding reactions were performed for 30 min at 37 °C in total volume of 100 µl with ConA final concentration at 9 µg/µl and DNA concentrations variable. The interrelation of DNA and ConA concentrations was established as followed: Firstly, the concentration of DNA during the incubation with ConA was variable (from 200 to 0.391 ng/µl), but the amount applied to the gel was the same for each sample (150 ng). Secondly, the concentration of ConA during the incubation with DNA was the same for each sample, but the ConA amount applied to the gel was variable (from 6.75 to 432 µg). The negative control contained no lectin. The DNA binding ability of the lectin under the described conditions was analyzed by electrophoresis and Southern-blot hybridization.

3 .6 Lectin-DNA-Binding at different pH Values

For the binding reactions, in which the influence of pH on the ConA-DNA-complex formation was examinated, lectin solutions and dilutions were prepared with 3 different buffers: 10 mM Tris-HCl pH 7.5, 20 mM acetate pH 6.0 and 20 mM acetate pH5.0. The concentrations of ConA, DNA and the incubation conditions were the same as already described for the binding assay (item 3 . 1 . 1).

3. 7 Removal of the protein content on the ConA-DNA-complex and recovery of the DNA

A treatment of the ConA-DNA-complex with protease in the presence of SDS and a subsequent extraction with phenol/chloroform was carried out to break down and remove the protein-content of the complex. For this purpose, ConA-DNA-complexes were allowed to form in binding buffer at 37°C for 30 min, using a fixed concentration of closed- or EcoRI-linearized plasmid-DNA (6 ng/µl) and 3 different ConA final concentrations (9, 1.2 and 0.16 µg/µl) in a total volume of 249 µl. In the control, DNA was incubated with binding buffer without ConA. After incubation, an aliquot of each sample was taken and analyzed by gel electrophoresis and southern blot hybridization. To the remaining volume of each sample, SDS 10% and proteinaseK (10 mg/ml) were added to give rise to 1% and 1 mg/ml final concentrations. The samples were incubated at 37°C for 2 h. After incubation, the volume of each sample was adjusted to 400 µl with TE-buffer and an equal volume of phenol/chloroform was added, followed by two additional extractions with chloroform. The obtained aqueous supernadante were submitted to precipitation at – 20°C by the addition of 3 M Na-Acetate pH5.2 and 100% EtOH and the pellets resuspended in TE-buffer. Finally, samples were analyzed by gel electrophoresis and southern blot hybridization.

3. 8 Lectin-DNA-Binding in the Presence of Metal Ions

For the lectin-DNA-binding reactions, in which the influence of metal ions (Ca^{2+}, Mn^{2+}, Mg^{2+}) was examinated, lectin and DNA concentrations were kept constant, while each metal ion was used at different concentrations. Typically, the total reaction volume of 30 µl contained 6 ng of DNA per µl, 1.2 µg lectin per µl and a final concentration of metal ions between 6 mM and 8.2 µM. Positive controls contained no metal ion, the negative controls no lectin.

3. 9 Effect of EDTA on the lectin-DNA-complex formation

3. 9. 1 Binding at constant EDTA concentration

For the binding reaction, in which the influence of low concentration of EDTA on the ConA-DNA-complex formation was examined, lectin stock solution, lectin dilutions and DNA working solution were prepared with TE-buffer (10 mM Tris-HCl pH7.5, 1 mM EDTA). The concentrations of ConA, DNA and the incubation conditions were the same as already described for the binding assay (item 3 . 1 . 1).

3. 9. 2 Binding in the presence of a variety of EDTA concentrations

To check if a broad range of EDTA concentrations would cause a proportional change on the ConA-DNA complex formation, ConA and DNA concentrations were kept constant, while EDTA solutions were used at different concentrations. A total reaction volume of 30µl contained 6 ng of DNA per µl, 1.2 µg lectin per µl, and a final concentration of EDTA between 60 mM and 0.1 µM. Positive controls contained no EDTA, the negative controls no lectin.

3. 9. 3 Binding after dialysis of the lectin against Tris-EDTA buffer

To investigate whether the EDTA-effect on the ConA-DNA-complex formation was reversible, ConA solution was first equilibrated by dialysis against an EDTA-containing buffer and then the dialyzed ConA solution incubated with DNA. The reversibility of the binding was tested by subsequent addition of either metal ions (set-1 experiment) or Tris-buffer (set-2 experiment).

A ConA-solution of 12 µg/µl was prepared in TE-buffer and transferred to a TE-buffer-equilibrated dialysis membrane. The ConA solution was extensively dialyzed against TE-buffer at 4°C for 3 days. After dialysis, the concentration of the dialyzed ConA solution was determined by measuring the absorbance at 280 nm wavelengths, taking the specific Extinction Coefficient of this lectin as reference (Agrawal and Goldstein, 1967a).

Serial dilutions of the lectin were prepared from the dialyzed ConA solution in TE-buffer. Binding reactions were performed in a total volume of 100 µl by adding DNA at 10 ng/µl to lectin samples at different concentrations. While the DNA concentration in the ConA-DNA mixtures was kept constant, the ConA concentrations were respectively 9, 4.5, 2.25 , 1.125, 0.562, 0.281, 0.141, 0.070, 0.035, 0.018, 0.009, 0.0045, 0.0022, 0.0011 and 0.0005 µg/µl.

As negative control, the DNA was mixed with TE-buffer devoid of lectin. After the ConA-DNA mixtures and the negative control were incubated at 37°C for 30 min, each one of them was split into two aliquots (set-1 and set-2 experiments). In set-1 experiment, $MgCl_2$ and $CaCl_2$ were added to each sample at 4 mM final concentration, while in set-2 experiment only binding buffer was added. All samples were incubated at 37°C for 30 min and subsequently centrifuged at 13,000 rpm for 5 min. The formation of precipitate could be observed by the presence of pellet in some samples. While the pellets were discarded, the supernadante were used for further analysis. Samples were, after addition of blue marker, loaded on agarose gels and analyzed by electrophoresis.

3. 10 Influence of Ionic Strength on Lectin-DNA-complex Formation

To investigate the influence of high ionic strength on the binding of ConA to DNA, two sets of experiments were carried out. In the first set, 1.2 µg/µl of ConA were incubated with 6 ng/µl at 37°C for 30 min, followed by an addition of NaCl to give arise to 500, 250, 125, 62.5 and 31.25 mM NaCl final concentrations. Samples were reincubated at 37°C for another 30 min and finally analyzed by gel electrophoresis and southern blot hybridization. In the second set of experiments lectins were first incubated with the NaCl solutions and afterward DNA was added. In the positive controls, no NaCl was added and, in the negative controls, DNA was incubated in the presence of 500, 125 and 31.2 mM NaCl.

3. 11 Hemagglutination

3. 11. 1 Preparation of the erythrocytes

Human blood cells type 0 were fractionated by ficoll gradient centrifugation, the erythrocyte fraction was washed with PBSd twice and the cell pellet was resuspended to a 6% cell suspension in Tris-NaCl buffer (10 mM Tris, 150 mM NaCl).

3. 11. 2 Lectin-DNA-binding reaction

Three samples of ConA-DNA complexes were prepared in binding buffer by incubating a constant ConA amount (1.5 mg) with three different pEGFP-C1-DNA amounts (150 µg, 75 µg and 37.5 µg), in a total volume of 250 µl, at 37°C for 30 min. In the control, the same amount of

ConA was incubated without DNA. Serial dilutions of the ConA-DNA-complexes and control were prepared in a 96-wells microtiter plate in binding buffer (50 µl dilution / well).

3. 11. 3 Addition of the erythrocytes and estimation of hemagglutination title

To each well of the plate, 50 µl Tris-NaCl buffer and 50 µl of the 6% erythrocyte suspension were added and the plates incubated at 25 °C for 1 h. After incubation, the results were recorded by photography.

3. 12 Agglutination of cell lines

3. 12. 1 Preparation of the cells

The human cell lines KB and A549 and the hamster cell line BHK21 were obtained from ATCC (American Type Culture Collection). Cells were grown in Dulbecco modified medium supplied with 10 % FCS. For the agglutination assays, monolayers of cells were washed twice with PBSd (PBS-buffer without Ca^{2+} and Mg^{2+} ions), cells were detached by incubation with trypsin/EDTA, centrifuged for 5 min, and the cell pellets were ressuspend in PBSd. Cells were finally ressuspended in Tris/NaCl-buffer (10 mM Tris, 150 mM NaCl, pH 7.5) at a cell density of 4.0 x 10^7 cells/ml.

3. 12. 2 Lectin-DNA-binding reaction

For the agglutination assay, dilution series of Herring sperm DNA containing 7,1395 to 55.8 ng/µl DNA in a total volume of 200 µl, were prepared in binding buffer in 24 well plates. A volume of 25 µl lectin at a concentration of 10 µg/µl was added and the plates were incubated at 37°C for 30 min. ConA was omitted in the negative controls, whereas the positive controls contained ConA, but no DNA.

3. 12. 3 Addition of the cells and estimation of the agglutination title

After incubation of the lectin-DNA-mixtures, a volume of 175 µl of Tris/NaCl-buffer (10 mM Tris, 150 mM NaCl, pH7.5) and 100 µl cell suspensions (4 x 10^7 cell/ml) were added and the plates were incubated at 25 °C for 1 h. In each well of the plate, the final concentration of lectin (0.5 µg/µl) and of cells (8 x 10^6 cells/ml) were constant, whereas the DNA-concentrations vary from 2,856 to 22.3 ng/µl. Negative control contained no ConA and positive control contained ConA, but no DNA. The agglutination of the cells was recorded by photography.

3. 13 Sephadex affinity chromatography of the ConA-DNA-complex

3. 13. 1 Chromatographic Procedure-1:

Sephadex G25 and Elution with Glucose Solution

The chromatographic procedure-1 was the first set of experiments designed to check the ability of ConA-DNA-complexes to bind to a glucose-based matrix and to establish optimal binding conditions. Using pre-packed Sephadex-G25 (Pharmacia NAP-10™) columns, it was possible to make a rapid evaluation of adequate chromatographic parameters.

A pre-packed Sephadex G25 (Pharmacia NAP-10™) 1.3 x 2.6 cm column was equilibrated with binding buffer, and the ConA-DNA mixture (800 µg ConA : 2.59 µg DNA), which had been preincubated at 37 °C for 30 min, was applied and allowed to bind to the column for 1 h at room temperature. The column was eluted first with four column volumes of binding buffer, and then a 0.1 M glucose solution was applied. Fractions were collected and their absorbance at 260 and 280 nm were measured. 45 µl Aliquots of each fraction were joined to stop 5 µl stop buffer (100 mM EDTA, 1% SDS, bromophenol blue / xylenocyol / glycerol) and loaded on an agarose gel. After electrophoretic separation, the DNA was transferred to nylon membranes and assayed by the Southern-blot hybridization technique.

3. 13. 2 Chromatographic Procedure-2:

Sephadex G75 and Elution with Glucose Gradient

Since basic parameters were already established in Procedure-1, the chromatographic procedure-2 was designed to investigate in greater detail the adsorbed ConA-DNA-complexes. Chromatographs in procedure-2 were carried out with a slower flow rate, using a Sephadex-G75 (Pharmacia) as matrix. Furthermore, adsorbed ConA-DNA-complexes were eluted with a broad range of either glucose or mannose gradients. Finally, chromatographic fractions were analyzed in two sets of electrophoresis to investigate the abundance of ConA-complexed- and unbound-DNA in the whole chromatography.

Sephadex G75 powder (Pharmacia) was dissolved in binding buffer and allowed to swell over night at 4 °C. The swollen Sephadex was packed into a 1 x 6.8 cm column and equilibrated with binding buffer. A ConA-DNA mixture (100 µg ConA : 1 µg DNA), which had been preincubated at 37 °C for 30 min, was applied to the equilibrated column and allowed to bind for

1 h at room temperature. The column was then eluted first with four column volumes of binding buffer, subsequently a continuous 0 to 0.5 M glucose gradient was applied. Fractions were collected and their absorbances at 260 and 280 nm were measured. From each fraction, 40µl-aliquots were reserved to two separated agarose gels. While for the first gel, the aliquots were joined to 5 µl of SDS-containing stop buffer (100 mM EDTA, 1% SDS, bromophenol blue / xylenocyol / glycerol), for the second gel aliquots received 5 µl of loading buffer (100 mM EDTA, bromophenol blue / xylenocyol / glycerol) . After electrophoretic separation, the DNA was transferred to nylon membranes and assayed by the Southern-blot hybridization technique.

3. 13. 3 Chromatographic Procedure 3:

Sephadex-G75 and Elution with Galactose and Mannose Solutions

Chromatographic procedure-3 was designed to check if the elution of ConA-DNA-complexes from Sephadex G75 columns was sugar specific. Chromatographs using procedure-3 were carried out with very long and thin Sephadex-G75 columns, which had show to be a critical parameter in optimal separation by affinity. As previous described in procedures-1 and 2, unbound material was eluted with binding buffer. Afterwards, galactose was applied, as a nonspecific sugar. For the elution of adsorbed ConA-DNA-complexes either glucose, mannose, α-methyl-D-glucopyranoside or α-methyl-D-mannopyranoside was applied. After each chromatography, fractions were analyzed in two sets of electrophoresis, to investigate the abundance of ConA-complexed- and unbound-DNA.

A Sephadex G75 (Pharmacia) 0.3 x 15 cm column was equilibrated with binding buffer, and the ConA-DNA mixture (600 µg ConA : 3 µg DNA), which had been preincubated at 37 °C for 30 min, was applied and allowed to bind to the column for 1 h at room temperature. The column was then eluted with four column volumes of binding buffer and subsequently with four volumes of a 0.1 M galactose solution. Finally, the column was eluted with 0.1 M mannose solution. Fractions were collected and their absorbances at 260 and 280 nm were measured. From each fraction, 60µl-aliquots were reserved for two separated agarose gels. While for the first gel, the aliquots were joined to 7 µl of SDS-containing stop buffer (100 mM EDTA, 1% SDS, bromophenol blue / xylenocyol / glycerol), for the second gel, aliquots received 7 µl of loading buffer (100 mM EDTA, bromophenol blue / xylenocyol / glycerol) . After electrophoretic separation, the DNA was transferred to nylon membranes and assayed by the Southern-blot hybridization technique.

3. 13. 4 Chromatographic Procedure-4:

Sephadex G75 and Elution with 0.1 M Glucose Solution

This chromatographic procedure was designed to test whether ConA-DNA-complexes, which were prepared with TE-dialyzed ConA (section 3. 9. 3), are able to bind to Sephadex. The Sephadex column (0.3 x 15 cm) was equilibrated with TE-buffer and the ConA-DNA-mixture (200 µg TE-dialyzed ConA : 1 µg DNA), which had been preincubated at 37 °C for 30 min, was applied. The ConA-DNA-mixture was allowed to bind to the Sephadex column for 1 h at room temperature. The column was then eluted first with four column volumes of TE-buffer, subsequently with 0.1 M glucose solution. Fractions were collected and their absorbances at 260 and 280 nm were measured. From each fraction, 40µl-aliquots were joined to 5 µl of SDS-containing stop buffer (100 mM EDTA, 1% SDS, bromophenol blue / xylenocyol / glycerol). After electrophoretic separation, the DNA was transferred to nylon membranes and assayed by the Southern-blot hybridization technique.

3. 14 Gel filtration chromatography of the ConA-DNA-complex

3. 14. 1 Column preparation

The pre-swollen Bio-Gel agarose (BioGel A 15m-fine, Bio-Rad) was packed in a 1 x 10 cm column support and extensively equilibrated against binding buffer. Then a flow rate of 0.15 ml/min was established and the column was ready to be loaded with the ConA-DNA-mixture.

3. 14. 2 ConA-DNA-binding reaction

The binding reaction was performed in binding buffer at 37°C for 30 min. A total volume of 200 µl contained 20 µg of DNA and 2 mg of ConA. Accordingly, the final concentration of DNA was 0.1 µg/µl and of ConA was 10 µg/µl.

3. 14. 3 Chromatographic fractions collection and estimation of peaks positions by absorbance measure

After incubation, the ConA-DNA-mixture was applied into the column and the elution with binding buffer was started. Fractions of 0.3 ml were collected in 2 min intervals and had their absorbances at 260 and 280 nm measured. The 260 and 280 nm absorbance measurements

were additionally used to calculate the relative protein and nucleic acid concentration of each fraction accordingly to the Warburg-Christian Concentration equation (Leyne, 1957).

Concentration Calculation Equations
Protein = abs(260)nm x (-757.29) + abs(280)nm x (1552.00)
Nucleic Acid = abs(260)nm x (62.90) + abs(280)nm x (-36.00)

3. 14. 4 Analysis of the collected fractions by electrophoresis and Southern-blot

After the absorbances at 260 and 280 nm of the fractions were determined, aliquots of 25µl were taken and joined with 4 µl of loading buffer (100 mM EDTA, bromophenol blue / xylenocyol / glycerol). Finally, samples were loaded on agarose gels for electrophoresis and Southern-blot hybridization.

3. 15 Hemagglutination assay of fractions of gel filtration chromatography in BioGel agarose

3. 15. 1 Chromatography and electrophoretic analysis of the fractions

A Bio-Gel agarose (BioGel A 15m-fine, Bio-Rad) 1 x 10 cm column was equilibrated with binding buffer, and the 200 µl ConA-DNA mixture (1000 µg ConA : 20 µg DNA), which had been preincubated at 37 °C for 30 min, was applied. The column was eluted with binding buffer and fractions of 0.3 ml were collected in 2 min intervals. After fractions collection, the absorbances at 260 and 280 nm were measured, and aliquots were taken for electrophoresis and Southern-blot hybridization.

3. 15. 2 Preparation of the human erythrocytes

Human blood cells type 0 were fractionated by ficoll gradient centrifugation, the erythrocyte fraction was washed with PBSd twice and the cell pellet was resuspended to a 6% cell suspension in PBSd buffer.

3. 15. 3 Serial dilution of the chromatographic fractions of peaks I and II

According to the absorbance measurements (item 3 . 15 . 1), representative fractions of peak I and peak II could be estimated. Serial dilutions of the peak I and II fractions were prepared in a 96-wells microtiter plate in binding buffer (50 µl dilution / well).

3. 15. 4 Addition of erythrocytes and estimation of the agglutination titer

To each well of the plate, 50 µl PBSd buffer and 50 µl of the 6% erythrocyte suspension were added and the plates incubated at 25 °C for 1 h. After incubation, the results were recorded by photography.

3. 16 Circular dichroism spectra

3. 16. 1 ConA-DNA-complex formation

The binding reaction was performed in binding buffer at 37°C for 30 min. A total volume of 300 µl contained 30 µg of DNA and 3 mg of ConA. Accordingly, the final concentration of DNA was 0.1 µg/µl and of ConA was 10 µg/µl.

3. 16. 2 Chromatography

A Bio-Gel agarose 1 x 10 cm column was equilibrated with binding buffer, and the ConA-DNA mixture was applied. The column was eluted with binding buffer and fractions of 0.3 ml were collected in 2 min intervals. After fractions collection, the absorbance at 260 and 280 nm were measured.

3. 16. 3 SDS-polyacrylamid gel electrophoresis analysis of peak I and peak II fractions

According to the absorbance measures (item 3 . 14 . 2), representative fractions of each peak were chosen to be analyzed by SDS-polyacrylamid gel electrophoresis. 37.5 µl of each fraction was joined to 12.5 µl blue marker and the total volume of 50 µl was applied to a 6 x 8 cm polyacrylamid gel (12%). The electrophoresis was performed at 30 mA for 2 h in 1 X running buffer. After staining with a Coomassie blue solution, the gel was incubated in destain solution and recorded by photography.

3. 16. 4 Circular Dichroism Spectroscopy (CD spectra)

The single components ConA and DNA, as well as the ConA-DNA-complex and peak I of BioGel agarose chromatography were used for the measures. A volume of 200 µl of ConA at 0.2µg/µl, 200 µl of DNA at 0.006 µg/µl and 400 µl of the mixture of both (ConA-DNA-complex) were used for incubation at 37°C for 30 min prior to the CD-measure. The sample of peak I corresponds to 200 µl of the chromatographic fraction 15. The same volume of binding buffer was applied as blank The spectrum measure was carried out with 200 µl of each sample in the range of 190 to 300 nm wave length at a velocity of 10 nm/min at 37 °C. CD measures were performed in a JASCO J-715 CD- spectropolarimeter using thermostatted quartz cells (1 mm optical path).

3. 17 Uptake of the lectin-DNA-complex by cells in culture

3. 17. 1 ConA-DNA-complex formation

The ConA-DNA complex formation was performed in binding buffer at 37°C for 30 min. A total volume of 300 µl contained 20 µg of DNA and 2 mg of ConA. Accordingly, the final concentration of DNA was 0.066 µg/µl and of ConA was 6.666 µg/µl.

3. 17. 2 Chromatography and electrophoretic analysis of fractions

A Bio-Gel agarose (BioGel A 15m-fine, Bio-Rad) 1 x 10 cm column was equilibrated with binding buffer, and the ConA-DNA mixture (2000 µg ConA : 20 µg DNA was applied. The column was eluted with binding buffer and fractions of 0.3 ml were collected in 2 min intervals. After fractions collection, the absorbances at 260 and 280 nm were measured, and aliquots were taken for electrophoresis.

3. 17. 3 Incubation of Peak I Fractions with Mammalian Cells in Culture

After absorbance measurements, peak-I fractions were chosen to be incubated with cells in culture. The volume of two fractions (approximately 500 µl) was joined to 1000 µl of PBSd. This procedure was necessary to avoid cell lysis during incubation. Mammalian cell lines (A-549, BHK21 and CHO), grown with Dulbecco medium (10% FCS) in 6-wells plates at 37°C, were first washed with PBSd and then the fraction/PBSd mixtures were transferred to each well of the plate. The plates were re-incubated at 37°C, 5% CO_2 for 2 hours. After incubation, cells

were washed with PBSd, treated with trypsin for detachment and transferred to 10 cm cell culture plates to grow in Dulbecco (10% FCS) at 37°C, 5% CO_2 for 24 h.

3. 17. 4 Analysis of cells by fluorescence microscopy

After 24 h incubation time, cells were washed twice with PBSd and directly analyzed by fluorescence microscopy in a Olympus fluorescence microscope. Green fluorescent cells were recorded by photography.

4. RESULTS

A possible interaction between leguminous lectins and DNA was investigated by incubating these biomolecules in solution and by analyzing the formation of lectin-DNA-complexes by electrophoretic mobility shift experiments (EMSAs). While lectin-DNA-complexes could be directly detected by gel electrophoresis, a more precise analysis of the DNA-shift pattern was performed by Southern transfer hybridization procedures. For the binding reactions, one type of plasmid-DNA (pEGFP-C1) and 13 different lectins, from 7 representative genera of the Leguminosae family, were used. The lectin of *Canavalia ensiformis* (ConA) was chosen as prototype for a detailed analysis of the lectin-DNA-complex formation.

4. 1 Formation of ConA-DNA-complexes

The lectin of *Canavalia ensiformis* (ConA) bound to DNA and formed ConA-DNA complexes as shown by electrophoretic mobility shift assays (EMSA). The migration of these complexes was retarded as compared to naked DNA, and the DNA-shift was dependent on the ConA concentration used (Fig. 4). A detailed analysis of the DNA-shift pattern in Fig. 4 revealed that the ConA-complexed DNA did not show discrete bands, such as supercoiled, nicked or oligomeric plasmid DNA-bands. This diffuse smear-like DNA migration was observed in all ConA concentrations. Although the extent of DNA binding of ConA was shown to depend on concentration, a maximum saturation point was not reached; a further increase in ConA concentration would not lead to a stronger shift in the migration of the complexed DNA.

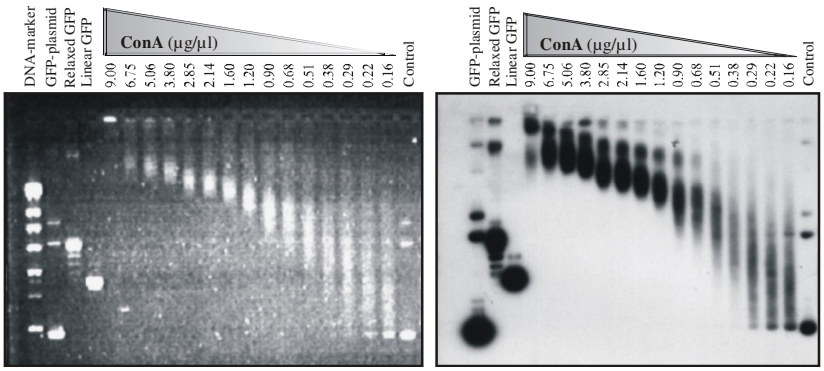

Figure 04: Analysis of ConA-DNA-complexes by EMSA and Southern transfer hybridizations. From the left to the right: Lambda-DNA-marker (26,282 to 421 bp), GFP plasmid and its relaxed and EcoRI-linearized forms. ConA concentrations from 9 to 0.16 µg/µl. In the controls, the plasmid was incubated in buffer devoid of lectins. The DNA from gels (UV-photographs on the left) was transferred to Nylon plus filters and hybridized to the ^{32}P-labeled pEGFP-C1 plasmid (Southern blots on the right). (Stoichiometric molar ratios ConA/DNA are presented in Appendix, Table 03).

4. 2 Partial inhibition of ConA-DNA-complex formation by methyl-α- D-glucopyranoside, or methyl-α-D-mannopyranoside

Specific sugars were used as competitors during the lectin-DNA binding reaction, to investigate whether the lectin-binding site for sugars was involved in the interaction with DNA. Since the vast majority of the lectins investigated were able to bind to glucose or mannose with high specificity and affinity (Reeke and Becker, 1988; Sharon and Lis, 1990; Sharon and Lis, 2002), derivates of these sugars were chosen for inhibition assays (see Appendix Table 01). The sugar galactose was used as a negative control, because all DNA-binding lectins were known to have very low affinity for this sugar.

The first inhibition assay was performed with the lectin of *Canavalia ensiformis* (ConA), which served as a model for further investigations. For the assay, different amounts of methyl-α-D-glucopyranoside, methyl-α-D-mannopyranoside or galactose were added to ConA prior to the addition of DNA (Fig. 5 A). A reduction in the shift of DNA migration was observed in the presence of methyl-α-D-glucopyranoside and methyl-α-D-mannopyranoside at all concentrations tested. This partial inhibition in the ConA-DNA complex formation was not dependent on the concentration of sugars (Fig. 5 A). Galactose shows no influence on the extent of the DNA shift, having no influence on the ConA-DNA complex formation. Moreover, the addition of any of these sugars after the ConA-DNA complex had formed, did not disrupt the interaction (Fig. 5 B).

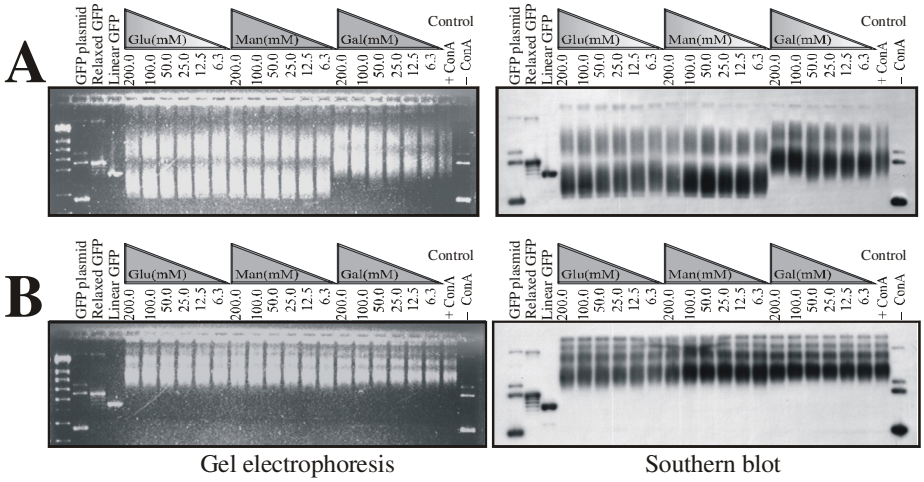

Figure 05: Partial inhibition of ConA-DNA-complex formation by specific sugars. (A) A reduction in the shift of DNA was achieved by pre-incubating ConA with methyl-α-D-glucopyranoside or methyl-α-D-mannopyranoside. The presence of galactose did not influence ConA-DNA-complex formation. (B) The addition of sugars after the ConA-DNA-complex had formed did not disrupt the complex.

4. 3 Adenosine-5'-triphosphate (ATP) inhibits ConA-DNA-complex formation

Most lectins bind to the monomers of specific sugars as well as to polymers of the same monomer (Reeke and Becker, 1988; Sharon and Lis, 1990). Since DNA is a polymer of deoxyribonucleotide units, it was interesting to investigate whether the deoxyribonucleotides of the DNA would also be recognized by the lectins. Furthermore, if lectins were to bind to deoxyribonucleotides, was conceivable that such deoxyribonucleotides served as inhibitors of the binding reaction between lectins and DNA. Adenosine-5'-triphosphate (ATP) was chosen to investigate this possibility.

The nucleotide inhibition protocol was first established for ConA and afterwards applied to the other lectins. For the ATP inhibition assay, ConA was incubated with different amounts of ATP before DNA was added. ATP inhibited ConA-DNA complex formation in a concentration-dependent manner (Fig. 6 A). This inhibition could only be observed where ATP was present during complex formation. ATP could not disrupt an already formed ConA-DNA complex (Fig. 6 B).

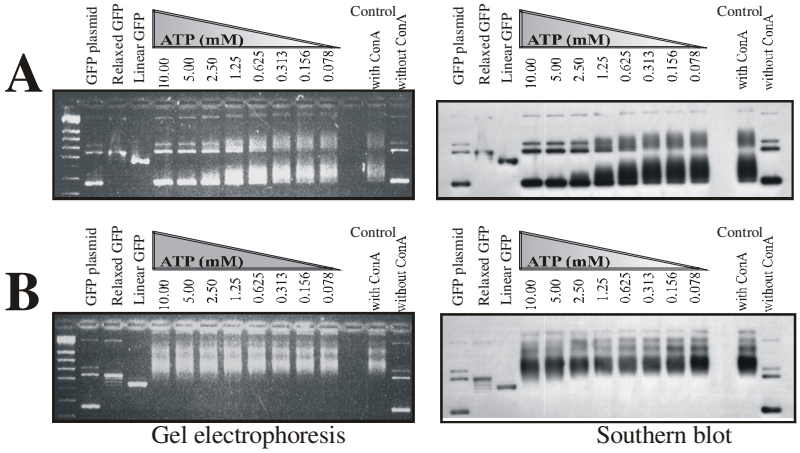

Figure 06: Adenosine-5'-triphosphate (ATP) inhibits ConA-DNA-complex formation. (A) The inhibition of the ConA-DNA-complex formation was achieved by pre-incubating ConA with ATP at different concentrations. (B) The addition of ATP after the ConA-DNA-complex had been formed did not disrupt the complex.

4. 4 No evidence for the inhibition of ConA-DNA-complex formation by 2-D-deoxyribose

The sugar binding specificity of ConA (see Appendix Table 01) did not include ribose or its derivatives (Agrawal and Goldstein, 1967a; Becker et al., 1975; Naismith and Field, 1996). Nevertheless, it was investigated whether the sugar component of DNA (2-D-deoxyribose) was able to bind to ConA and inhibit the lectin-DNA-complex formation. ConA was first incubated with various amounts of 2-D-deoxyribose and the DNA was added afterwards. None of the tested concentrations inhibited ConA-DNA complex formation under either experimental regime (Fig. 7).

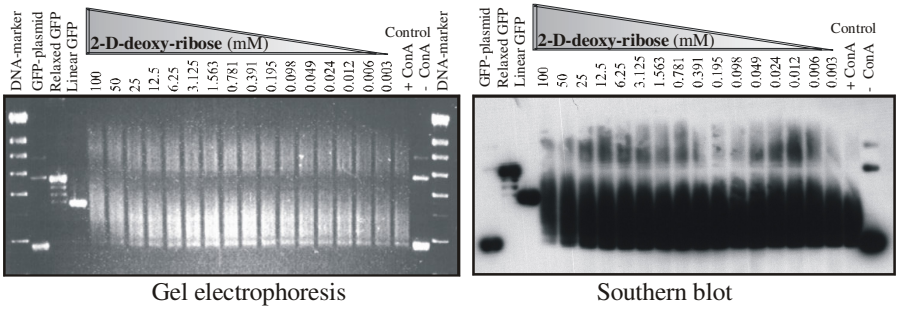

Figure 07: The sugar 2-D-deoxyribose does not inhibit ConA-DNA-complex formation. From left to right: DNA-markers, 2-D-deoxyribose concentrations from 100 to 0.003 mM and controls. The DNA from gel (UV-photography on the left) was transferred to a Nylon membrane and detected by hybridization (Southern blot on the right).

4. 5 Anti-ConA-antibody partially inhibits ConA-DNA-complex formation

A polyclonal anti-ConA antibody was used to block or diminish the ConA surface area available to interact with DNA. For the antibody-concentrations used, only a partial inhibition of lectin-DNA-complex formation was elicited (Fig. 8). This low efficiency of inhibition was understandable considering the antibody concentrations applied. In binding reactions, the ConA-final concentration was 1.2 µg/µl and the antibody concentrations varied from 0.278 to 0.00053 µg/µl. Therefore an excess of ConA was certainly present in all binding reactions. Interesting results were observed in samples with antibody concentration from 17.38 to 139 ng/µl. These samples showed a "supershift" at higher positions, which was probably the result of complexation of antibody-ConA-DNA.

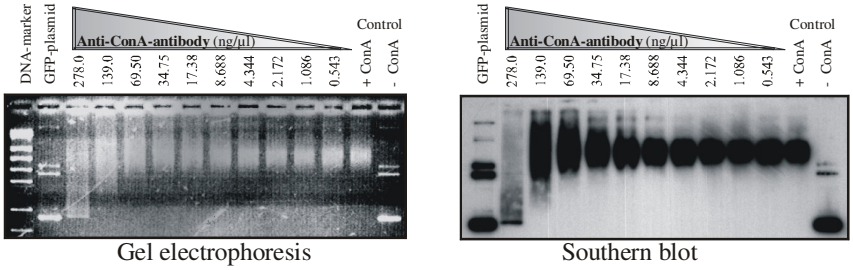

Figure 08: Partial inhibition of ConA-DNA-complex formation by anti-ConA-antibody. From left to right: DNA-markers, Anti-ConA-antibody concentrations from 278 to 0.543 ng/µl and controls. DNA from gel (UV-photography on the left) was transferred to Nylon membrane and detected by hybridization (Southern blot on the right).

4. 6 No evidence for sequence specificity of ConA-DNA-binding

Several subfragments of the plasmid pEGFP were used as binding partners for lectin-DNA complex formation to access a possible sequence contribution to the reaction. The linearized 4.741 bp plasmid was prepared by restriction with EcoRI, and smaller DNA fragments were produced by cleavage with HaeII. ConA shifted the migration of all DNA fragments tested in a concentration-dependent manner (Fig. 9). The migration of DNA fragments from the pUC-plasmid or from lambda-DNA were also bound and shifted by ConA. The interaction between ConA and the pEGFP-DNA did not appear to be sequence-specific, since this lectin was able to bind to the whole plasmid (Fig. 4) as well as to its subfragments (Fig. 9 A and B) and to DNA from other sources (Fig. 9 C).

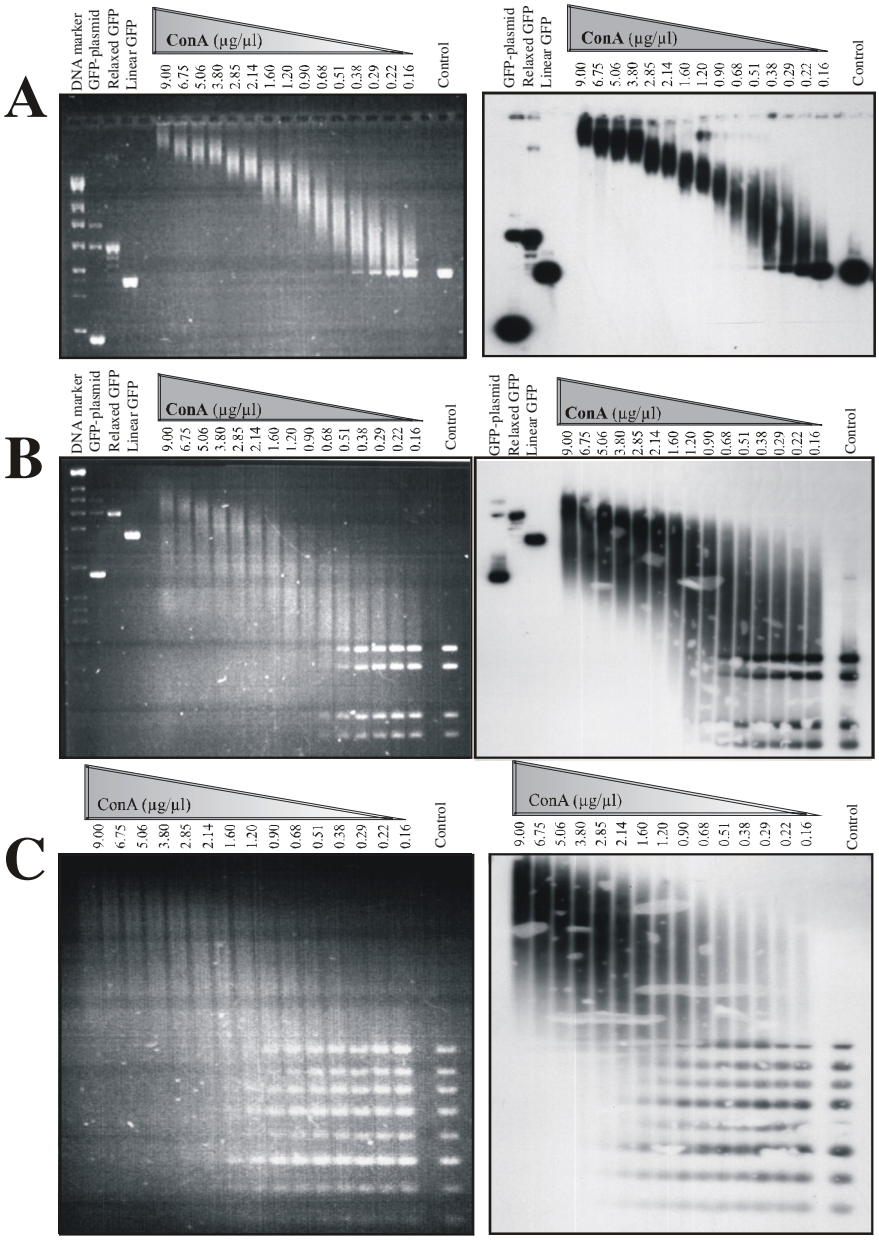

Figure 09: EMSA of complexes of ConA with pEGFP-C1 subfragments or DNA of other sources. (A) EcoRI-linearized pEGFP-C1 plasmid DNA, (B) Hae II subfragments of the pEGFP-C1 or (C) DNA from other sources bind to ConA. In C, a mixture of DNA fragments derived from pUC plasmid, bacteriophage lambda and yeast DNA fragments were used as binding partner. (Stoichiometric molar ratios ConA/DNA are presented in Appendix, Table 03 and 05).

4. 7 Kinetics of ConA-DNA-complex formation

An analysis of the time course of lectin binding to DNA might reveal details on the affinity of these binding partners to each other. An attempt to follow the kinetics of binding was first performed by measuring lectin-DNA-complex formation at 30 min intervals. Since saturation of binding was already reached during the first hour of the reaction, further experiments were performed with intervals in a range of minutes, e. g. ConA-DNA-complex formation within 3 min (Fig. 10 A) or in 1 min intervals (Fig. 10 B). The appearance of the DNA-shift after the first minute of reaction attested to very rapid binding of ConA to DNA. The results indicated that the ConA-DNA-binding occurred in a matter of seconds. Moreover, an extension of these measurements to hourly intervals had shown that the lectin-DNA-binding remained stable up to 24 h at room temperature.

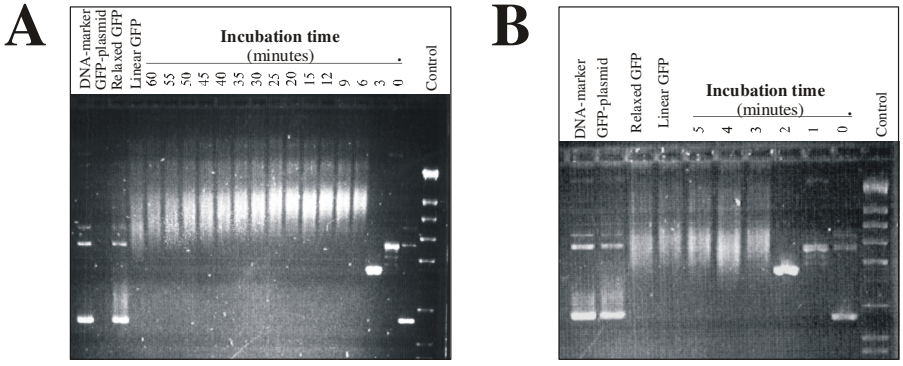

Figure 10: **Kinetics of ConA-DNA-complex formation**. EMSA of ConA-DNA-complexes at time intervals of 3 min (A) and of 1 min (B). From left to right: Control, samples of different time points of incubation and DNA-markers.

4. 8 Influence of DNA concentration on the size of ConA-DNA-complexes

In binding experiments designed to examine the relationship between the amount of lectin and the degree of lectin-DNA-complex formation, the DNA-concentration was kept constant and the amount of lectin varied (Fig. 4 and 9). Would a variation on DNA concentration and maintenance of the amount of ConA influence complex formation to the same extent? To answer this question ConA-DNA-complexes were formed using a single ConA concentration (9 µg/µl) and decreasing DNA concentrations (from 200 to 0.391 ng/µl). In the control sample, the pEGFP-C1 plasmid-DNA migrated to the expected position (Fig. 11 B and D); in samples containing ConA, most of the complexed DNA was retained in the gel pouches (Fig. 11 A and C). The results indicated that the size of the ConA-DNA-complex did not vary much for high ConA-concentration and variable DNA amount. The vast majority of the ConA-complexed DNA

was probably too big to enter the gel. Since the range of separation in 0.8% agarose gels is approximately 0.8 – 10 Kb, the size of the ConA-DNA-complexes has probably exceeded that limit (Sambrook et al., 1989). Nevertheless, the small amount of DNA that entered the gel migrated as a smooth smear and the extent of its migration was proportional to the DNA-concentration during complex formation. Interestingly, in those samples where almost all DNA sticked to the gel slots, the excess of non-bound ConA entered the gel and migrated to lower positions in the gel (right site of Fig. 11 A). The unbound ConA was stained with ethidium bromide in the gel (Fig. 11 A), but did not gave rise to hybridization signals in the Southern blot hybridization experiment (Fig. 11 C).

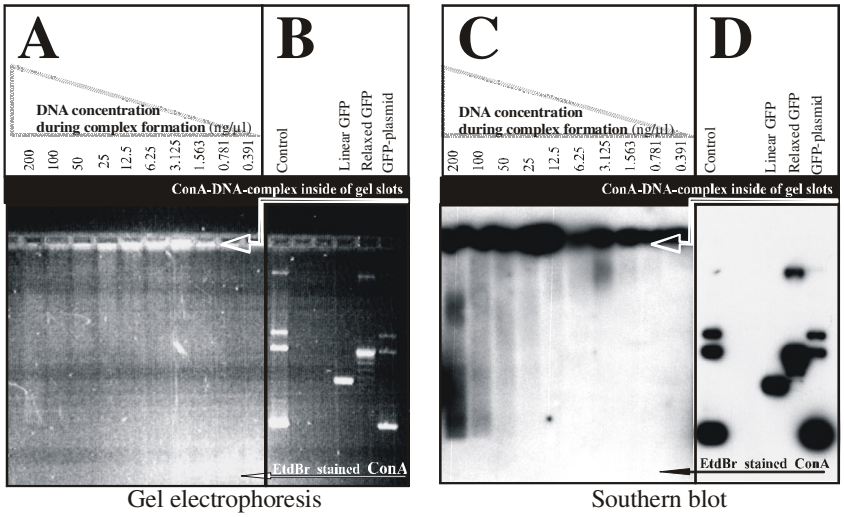

Figure 11: Influence of DNA-concentration on the size of the ConA-DNA-complexes. (A) and (B) EMSA; (C) and (D) Southern Blot. ConA-DNA-complexes were allowed to form by incubating a constant amount of ConA with different amounts of DNA. After incubation, equivalent amounts of DNA were applied to each slot of the gel, and ConA-DNA-complexes were analyzed by electrophoresis. From left to the right: Samples obtained by complex formation using DNA-concentrations from 200 to 0.391 ng/µl, control sample (without ConA) and DNA-markers. The photographs represented in (C) and (D) derived from two different exposition times of the Kodak films to the P^{32}-hybridized Southern blot. (Stoichiometric molar ratios ConA/DNA are presented in Appendix, Table 06).

4. 9 ConA-DNA-complex formation at different pH values

The conformation of ConA and its dimer/tetramer equilibrium are highly dependent on the pH of the solution (Mc Kerzie and Sawyer, 1973; Senear and Teller, 1981a). While at pH values higher than 7.0, ConA presents a tetrameric conformation, at pH values lower than 5.0, the dominant conformation is the dimeric. For the pH range between 5 and 7, dimeric and tetrameric ConA are in equilibrium (Senear and Teller, 1981b).

Binding reactions were performed in solutions at 3 different pH values, to investigate, whether the conformation of ConA would influence the formation of ConA-DNA-complexes. The formation of the ConA-DNA-complexes was pH-dependent (Fig. 12). While at pH 7.5 the lectin-DNA complex was well developed, it was much weaker at pH 6.0 and almost not apparent at pH 5.0. The results indicated that the extent of DNA-shift was proportional to the pH of the solution during the binding reaction.

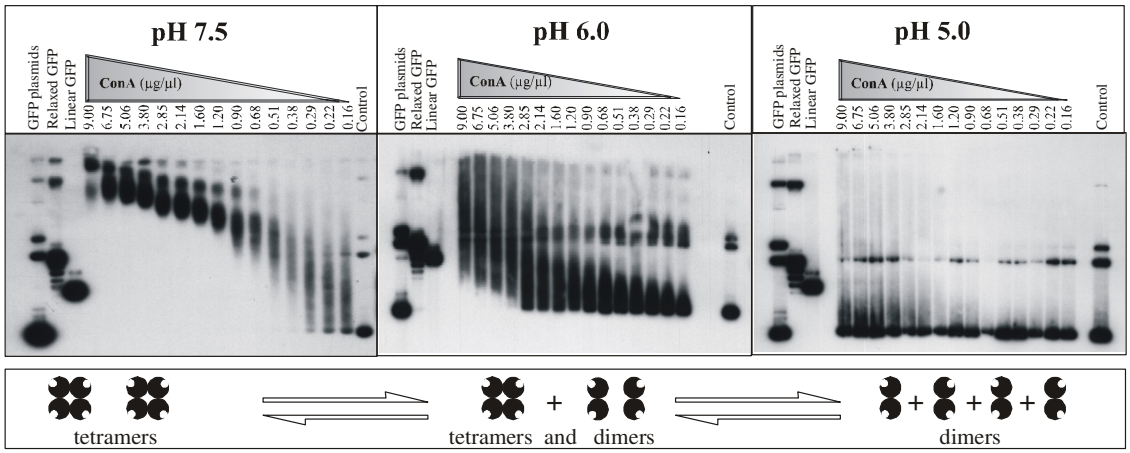

Figure 12: ConA-DNA-complex formation at different pH values. Different amounts of ConA were incubated with pEGFP-C1 DNA at pH 7.5 (Tris 10mM pH7.5), pH 6.0 (20 mM acetate pH6.0) or pH 5.0 (20 mM acetate pH5.0). The ConA-DNA-complexes were analyzed by EMSA and by Southern-transfer hybridization. (Stoichiometric molar ratios ConA/DNA are presented in Appendix, Table 03).

4. 10 DNA is retrieved intact from the ConA-DNA-complex after proteolysis

The DNA in the ConA-DNA-complex could be recovered intact upon SDS-proteinaseK treatment of the complex and by phenol-chloroform extraction (Fig. 13). Subsequently, the liberated DNA migrated in the gel like untreated DNA (Fig. 13 B). Even when closed supercoiled circular plasmid DNA was used for complex formation, most of the DNA re-isolated upon proteolysis could be recovered intact (Fig. 13, lanes 1 - 3, left panels). The linearized plasmid could be retrieved almost completely (Fig. 13, right panels). Hence, there was no evidence that the DNA was markedly degraded during complex formation with ConA.

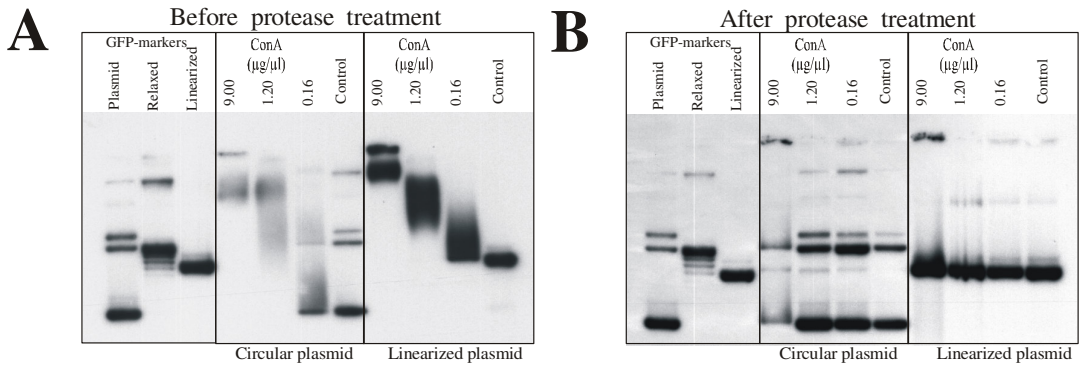

Figure 13: **Recovery of the pEGFP-DNA from the complex**. (A) The circular or the linearized pEGFP-C1 DNA was incubated with 3 different concentrations of ConA, and aliquots of the ConA-DNA-complex were analyzed by EMSA. (B) Aliquots of the ConA-DNA-complex were treated with SDS and proteinase K, and the protein was removed by phenol-chloroform extraction. After EtOH-precipitation, the DNA was solubilized in TE buffer and analyzed by EMSA and by Southern-transfer hybridization. (Stoichiometric molar ratios ConA/DNA are presented in Appendix, Table 07).

4. 11 The effect of metal ions

An excess of the metal ions Ca^{2+}, Mn^{2+} or Mg^{2+} in the reaction mixture during the incubation of ConA with DNA considerably reduced the binding of ConA to DNA (Fig. 14). This reduction was reflected in a weaker shift of the DNA in the EMSAs or no shift at all. This inhibitory effect was already apparent at metal ion concentrations even below 1 mM. ConA could bind to DNA with the same efficiency as in the control when the concentration of metal ions was below 0.03 mM.

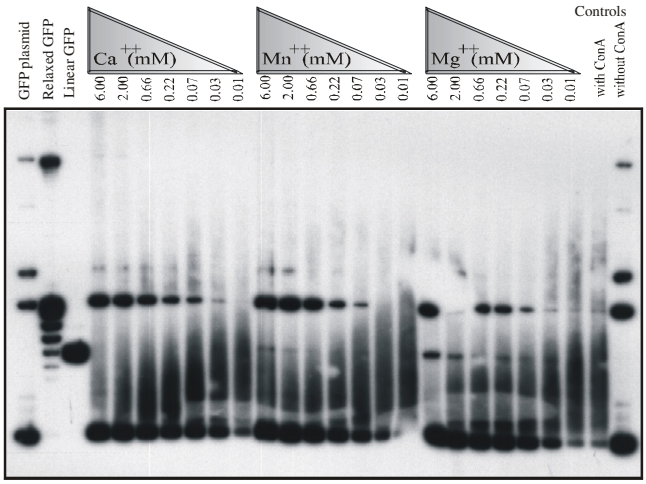

Figure 14: **Effect of metal ions on ConA-DNA-complex formation**. The binding of ConA to pEGFP-C1 DNA was apparent in the absence of metal ions (controls on the right) and decreased in the presence of increased concentrations of Ca^{++}, Mn^{++} and Mg^{++}. The DNA was analyzed by EMSA and by Southern-transfer hybridization.

4. 12 The effect of EDTA

4. 12. 1 EDTA present during ConA-DNA-complex formation

ConA could bind to DNA in the presence of 1 mM EDTA (Fig. 15 A). The extent of the observed DNA-shift was dependent on the concentration of ConA. Moreover the pattern of the DNA-shift was distinguishable from that obtained in the absence of EDTA (Fig. 4). In the presence of EDTA, the different DNA conformations (supercoiled, nicked and oligomeric) were shifted more independently, giving rise to more distinguishable shifts (Fig. 15 A). This effect was more striking in the presence of EDTA at concentrations between 1.975 and 0.0073 mM (Fig. 15 B). EDTA concentrations below 0.0073 mM did not influence the formation of the ConA-DNA complex, while concentrations higher than 15 mM completely abolished complex formation (Fig. 15 B).

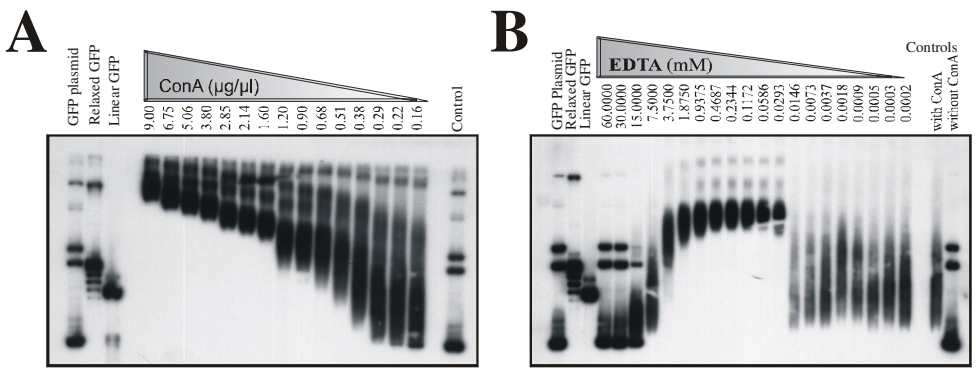

Figure 15: Effect of EDTA on the ConA-DNA-complex. (A) EMSA of the ConA-DNA-complex in the presence of 1 mM EDTA (Stoichiometric molar ratios ConA/DNA are presented in Appendix, Table 03). (B) Influence of different EDTA concentrations on the formation of the ConA-DNA-complex. (Stoichiometric molar ratio ConA/DNA is presented in Appendix, Table 04).

4. 12. 2 ConA dialyzed against EDTA and the formation of ConA-DNA-complexes

The results shown in Fig. 15 indicated that poorly metallized ConA bound better to DNA and yielded stronger DNA-shifts. Hence, an excess of metal ions led to lower ConA-binding to DNA and to a reduction in ConA-DNA-complex formation (Fig. 14). The question arose of whether the addition of metal ions to poorly metallized ConA would compromise the ability of ConA to bind to DNA. To approach this question, ConA solution was dialyzed against TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and used for the DNA-binding reaction with or without the addition of metal ions. As expected, TE-dialyzed ConA was able to bind to DNA in a

concentration-dependent manner (Fig. 16 A, right). When the TE-dialyzed ConA was incubated with DNA in the presence of metal ions, no ConA-DNA-complex was formed (Fig. 16 A, left). This observation reinforced the notion that metallized ConA did not bind to DNA. Would the addition of metal ions then disrupt an already formed ConA-DNA-complex? TE-dialyzed ConA was used for the incubation with DNA and metal ions or binding buffer were added to the ConA-DNA-complex after its formation. ConA-DNA-complex stability was not altered by the addition of binding buffer (Fig. 16 B), but by that of metal ions (Fig. 16 C). The addition of metal ions resulted in a massive precipitation of the ConA-DNA-complex and the precipitate could not be loaded on the gel. Actually, this co-precipitation effect indicated that the ConA-DNA-complex could not be disrupted by the addition of metal ions. In summary, the results presented in Fig. 14, 15 and 16 confirmed the impact of metal ions on ConA-DNA-complex formation and stability.

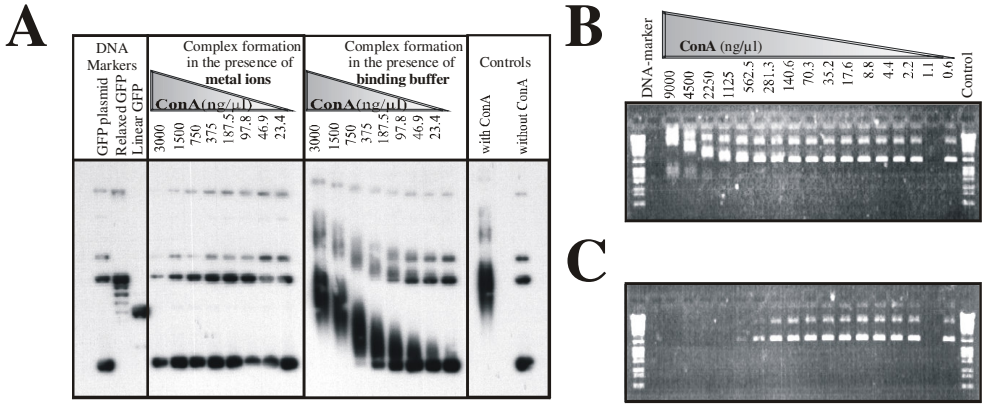

Figure 16: DNA-binding ability of TE-dialyzed ConA and stability of the ConA-DNA-complexes. (A) Formation of ConA-DNA-complexes using TE-dialyzed ConA in the presence (on the left) or absence (on the right) of metal ions. EMSA analyses of ConA-DNA-complexes after addition of binding buffer (B) or metal ions (C). In the left site of (C), the disappearance of DNA in the first five samples was due to spontaneous precipitation after metal ions addition. (Stoichiometric molar ratios ConA/DNA are presented in Appendix, Table 08 and 09).

4. 13 Disruption of the ConA-DNA-complex at high ionic strength

The stability of the ConA-DNA-complex was investigated by adding salt (NaCl) at different concentrations after the ConA-DNA complex had formed (Fig. 17 B) or by treating ConA with the same NaCl concentrations before the addition of DNA (Fig. 17 C). The binding of ConA to DNA was highly dependent on the ionic strength during incubation. The presence of salt strongly reduced or even inhibited complex formation (Fig. 17 C). Moreover, the addition of salt at concentrations higher than 250 mM after the ConA-DNA complex had formed, disrupted the interaction (Fig. 17 B).

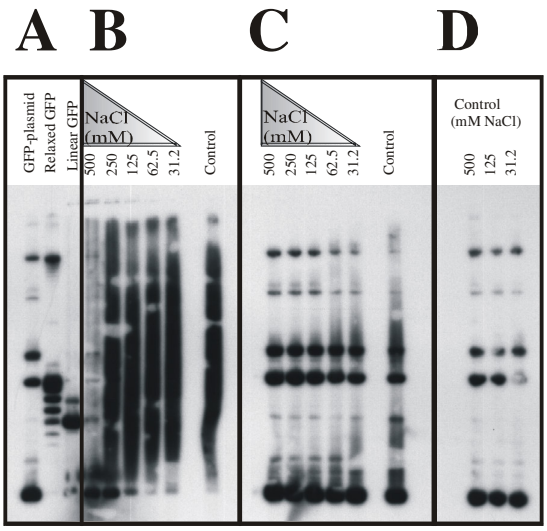

Figure 17: Influence of ionic strength on the formation and stability of ConA-DNA-complexes. (A) pEGFP-C1 DNA-markers: plasmids, relaxed and linearized. (B) The addition of salt (500 mM) after the ConA-DNA complex had formed caused the disruption of the complex. (C) The pre-incubation of ConA with salt reduced or even inhibited ConA-DNA-complex formation. In the controls (D), DNA was incubated with different concentrations of salt in the absence of ConA.

4. 14 Detection of hemagglutination after ConA-DNA-complex formation

The natural ability of ConA to agglutinate erythrocytes is due to the sugar-binding site of this lectin (Noonan and Burger, 1973; Huet, 1973; Schnebli and Bächi, 1975; Vlodavsky and Sachs, 1975). Accordingly, specific sugars acting on ConA are able to inhibit this agglutination. Since sugars specific for ConA reduced the formation of ConA-DNA-complexes (Fig. 5), it would be worth knowing whether DNA-complexed ConA was still able to bind and agglutinate cells. To investigate the influence of DNA on the ability of ConA to agglutinate erythrocytes, this lectin was first incubated with 3 different amounts of DNA, and the ConA-DNA-complexes were serially diluted and exposed to erythrocytes. As a control, ConA was used for agglutination without DNA. Erythrocytes were still agglutinated in the presence of all 3 ConA-DNA complexes (Fig. 18). The extent of agglutination depended on the molecular ratio ConA/DNA. The higher the ConA/DNA-ratio was, the more the erythrocytes were agglutinated. The binding of ConA to DNA did not inhibit agglutination, but reduced the agglutination titer one fold for a ConA/DNA-ratio of 2,274 and two fold for ConA/DNA-ratio of 1,137. This result indicated that the DNA-binding to ConA compromised to a certain extent the ability of ConA to agglutinate erythrocytes and that the degree of the influence on DNA-binding was dependent on the molecular ratio of ConA/DNA.

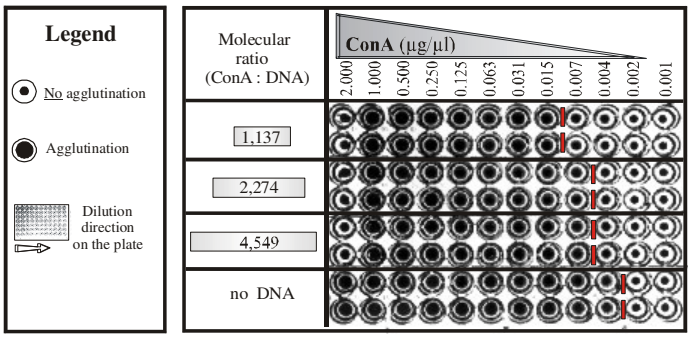

Figure 18: Hemagglutination after ConA-DNA-complex formation. ConA-DNA-complexes at 3 different ConA/DNA molecular ratios were prepared, incubated and afterwards serially diluted on a microtiter plate (direction of dilution: from the left to the right). After serial dilution and the addition of erythrocytes, the final ConA concentrations (2.000 to 0.001 µg/µl) corresponded to those shown in the panel. The control contained ConA, but no DNA.

4. 15 Mammalian cell lines can be agglutinatinated after ConA-DNA-complex formation

Previous results indicated that the formation of ConA-DNA-complexes reduced the extent of ConA-induced hemagglutination (Fig. 18). Would high concentrations of DNA completely abrogate ConA-induced agglutination? Experiments were performed to investigate the influence of different amounts of DNA on the ability of ConA to agglutinate mammalian cells. ConA, at a constant concentration, was mixed with various amounts of DNA, allowing the formation of ConA-DNA-complexes with a broad range of ConA/DNA-ratios. Subsequently, mixtures were exposed to mammalian cell suspensions. Mammalian cell lines could be agglutinated after the formation of ConA-DNA-complexes in all mixtures tested (Fig. 19). The results indicated that the agglutination of cells in the presence or absence of DNA was very similar. To estimate the influence of DNA-binding on the ConA-induced agglutination it would be necessary to serially dilute every ConA/DNA mixture after ConA-DNA-complex formation. Such estimation was exemplified in the agglutination of erythrocytes in section 4. 14 (Fig. 18).

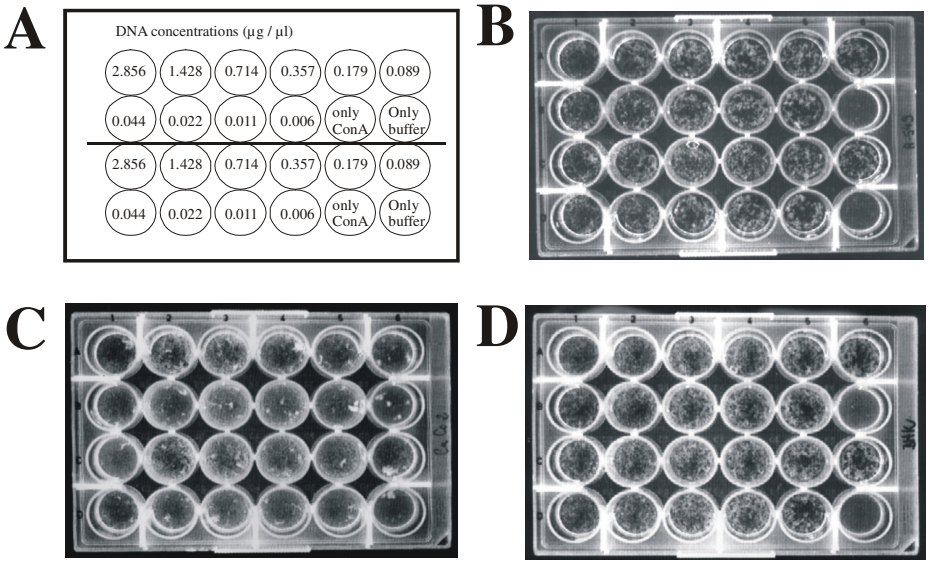

Figure 19: **Cell agglutination after ConA-DNA-complex formation**. (A) Panel of concentrations for agglutination of cell lines. (B) Human lung carcinoma, cell line A549. (C) Human enterocytes, cell line CaCo-2. (D) Baby hamster kidney cells, cell line BHK.

4. 16 ConA-DNA-complexes can bind to a Sephadex G 75 column, and bound complexes can be eluted by specific sugars

Chromatographic procedures were applied to test the ability of the ConA-DNA complex to bind by affinity to a specific sugar-based matrix. In the first set of experiments, ConA-DNA-complexes were applied to Sephadex G25 columns, fractions were collected and their absorbances at 260 and 280 nm were measured. Fractions eluted from the column were then applied to agarose gels, separated by electrophoresis and blotted to nylon membranes as described under Material and Methods. ConA-DNA complexes could bind to a Sephadex G25 column and could be eluted with 0.1 M glucose solution (Fig. 20 A). The Southern-blot of the chromatographic fractions confirmed the presence of DNA in peak I as well as in peak II. The abundance of ConA-complexed DNA in peak II was considerable higher than in peak I (Fig. 20 B). Although the absorbance in peak I was much higher than in peak II, the hybridization signals in peak II were stronger. Therefore, it was possible that the absorbance of ConA-DNA-complexes differs from the mere addition of absorbances due to ConA and DNA.

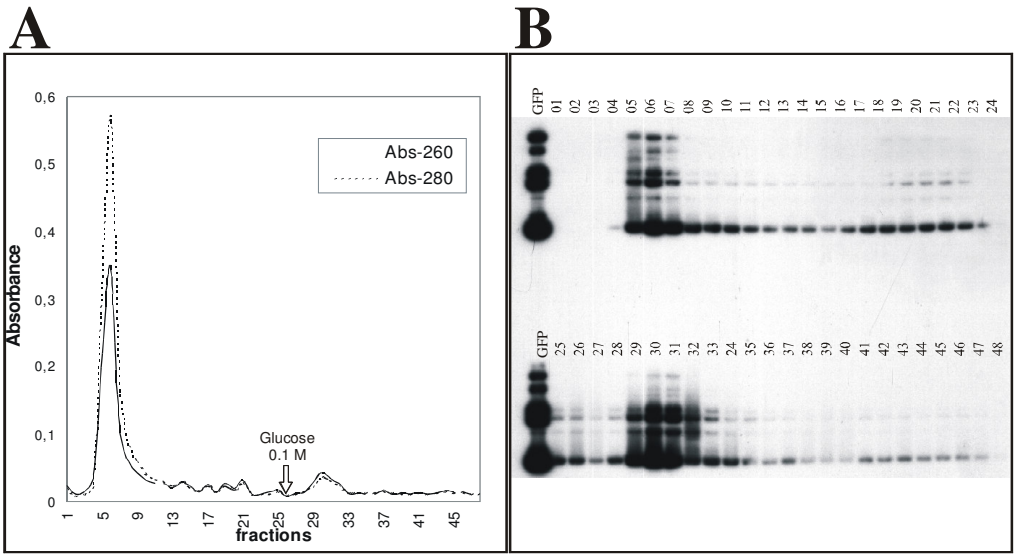

Figure 20: **Sephadex G25 affinity chromatography of the ConA-DNA complex**. (A) Chromatographic profile of fractions with absorbance measurements at 260 and 280 nm. (B) Southern blot hybridization analyses of individual fractions.

The chromatography on Sephadex G75 shown in Fig. 21 exemplified the occurrence of ConA-DNA-complexes in peak I and peak II, and determined the range of glucose concentrations at which the Sephadex-bounded ConA-DNA-complexes were effectively eluted. The Southern blot experiment demonstrated that the abundance of ConA-complexed DNA in peak I was higher than in peak II (Fig. 21 B).

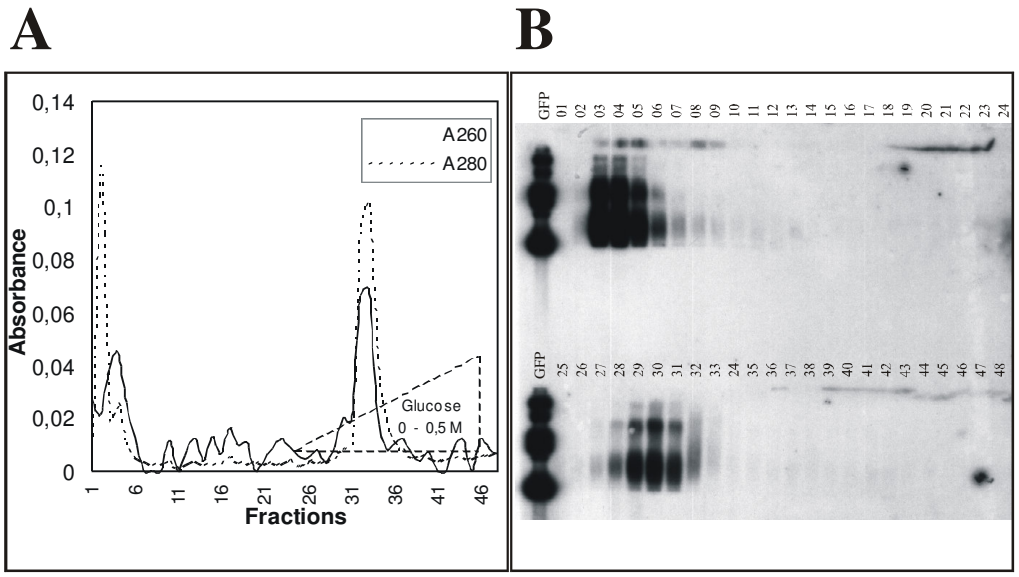

Figure 21: **Sephadex G75 affinity chromatography of the ConA-DNA complex**. (A) Chromatographic profile of fractions with absorbance measurements at 260 and 280 nm. (B) Southern blot hybridization analyses of the individual fractions.

The chromatography data illustrated in Fig. 22 confirmed the sugar specificity of the ConA-DNA-complex binding to the Sephadex G 75 column. The elution of the ConA-DNA complex from the Sephadex G75 column was sugar specific, since galactose solutions could not elute the complex, whereas glucose did (Fig. 22).

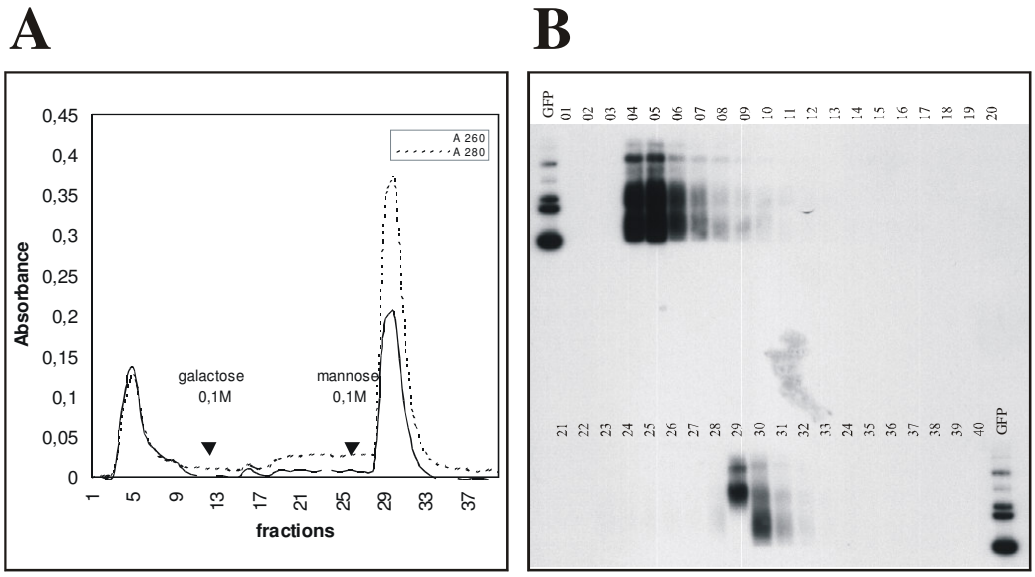

Figure 22: Sephadex G75 affinity chromatography of the ConA-DNA-complex. (A) Chromatographic profile of fractions with absorbance measurements at 260 and 280 nm. (B) Southern blot hybridization analyses of the individual fractions.

The ConA molecule must be at least partially metallized in order to bind to the Sephadex column (Agrawal and Goldstein, 1967b). Therefore a ConA solution that had been extensively dialyzed against TE-buffer (10 mM Tris, 1 mM EDTA, pH7.5) would not be able to bind to a Sephadex column (Karlstam, 1973; Stark and Sherry, 1979; Hardman et al., 1982). A ConA-DNA-complex was prepared with the poorly metallized ConA after dialysis against TE-buffer (Fig. 16) and an affinity chromatography on Sephadex G75 was performed. Under these conditions, the ConA-DNA-complex did not bind to the column (Fig. 23). This finding supports the notion that poorly metallized ConA is able to form a complex with DNA, however the obtained ConA-DNA-complexes did not bind to a Sephadex column.

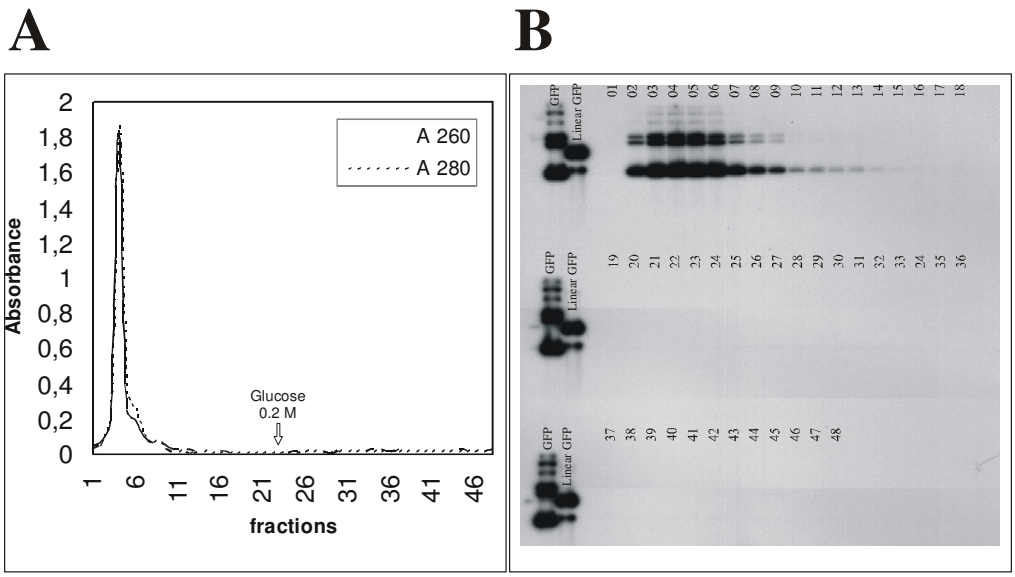

Figure 23: Sephadex G75 affinity chromatography of the ConA-DNA-complex, which was prepared with TE-dialyzed ConA. (A) Chromatographic profile of fractions with absorbance measurements at 260 and 280 nm. (B) Southern blot hybridization analyses of the individual fractions.

4. 17 ConA-DNA-complexes and free ConA can be separated by gel filtration on BioGel agarose columns

ConA-DNA-complexes were prepared by incubating various amounts of ConA to a given DNA concentration (Fig. 4 and 9). Since the extent of complex formation was directly proportional to the ConA concentration and no saturation point of binding could be reached, it was plausible to presume that part of the ConA entered into the reaction remained as free ConA. Free ConA was therefore separated from DNA-complexed ConA by gel filtration on a BioGel agarose column. The profile of this gel filtration as well as the Southern blot analysis of its fractions were presented in Fig. 24. The filtration chromatography resolved the whole ConA-DNA-complex in two distinguishable peaks with different absorbance patterns. While peak I fractions predominantly absorb at 260 nm, peak II fractions yielded higher absorbance at 280 nm (Fig. 24 A). Southern blot analysis revealed that ConA-DNA-complexes were only present in peak I. The excess of ConA was found in peak II (Fig. 24 A). Moreover, peak II was much greater than peak I. Hence the bulk of the ConA present during the complex reaction remained as free ConA in solution.

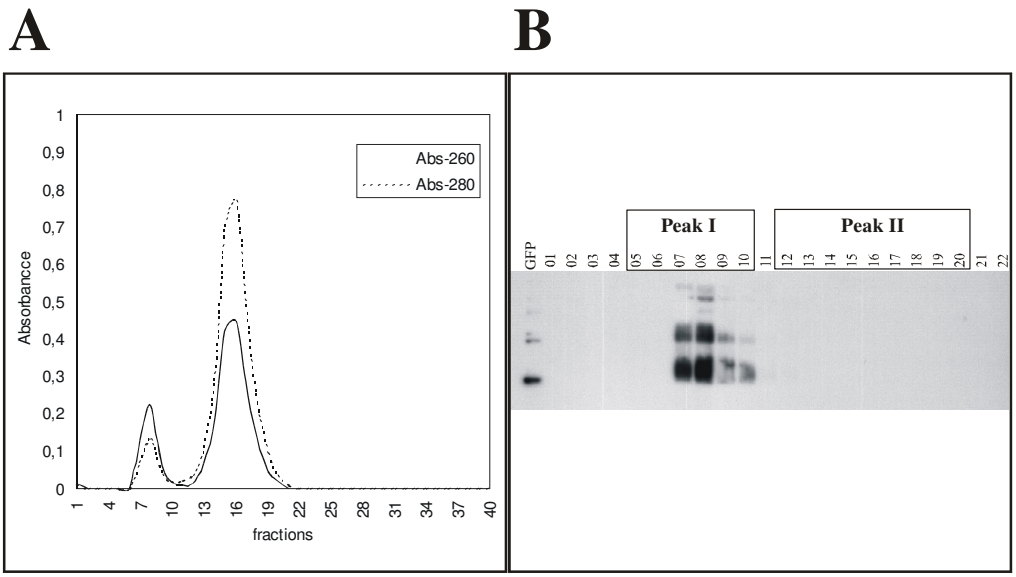

Figure 24: BioGel agarose filtration chromatography of the ConA-DNA-complex. (A) Chromatographic profile of fractions with absorbance measurements at 260 and 280 nm. (B) Southern blot hybridization analyses of the individual fractions.

4. 18 Hemagglutination of fractions from gel filtration on BioGel agarose

The observation that erythrocytes could be agglutinated in the presence of ConA-DNA complexes, and that the extent of agglutination depended on the molecular ratio of ConA/DNA (Fig. 18), raised the question, how much of the DNA-complexed ConA would be directly involved in the agglutination process. BioGel agarose chromatography afforded separation of DNA-complexed ConA (peak I) from free ConA (peak II) (Fig. 24). Therefore, the influence of DNA-complex formation on ConA-derived agglutination could be assessed by testing the ability of peak I and peak II fractions to cause hemagglutination. The chromatographic profile represented in Fig. 25 presented the separation of the two peaks as well as the distribution of their hemagglutination activities. Peak I fractions, which contained ConA-DNA-complexes, lacked hemagglutination activity (Fig. 25 A and B). The whole hemagglutination activity detected corresponded to the fractions in peak II. These data demonstrated that ConA-DNA-complexes (peak I) were unable to agglutinate erythrocytes, and that the hemagglutination detected was due to the presence of remaining free ConA (peak II), which had not entered into complex formation.

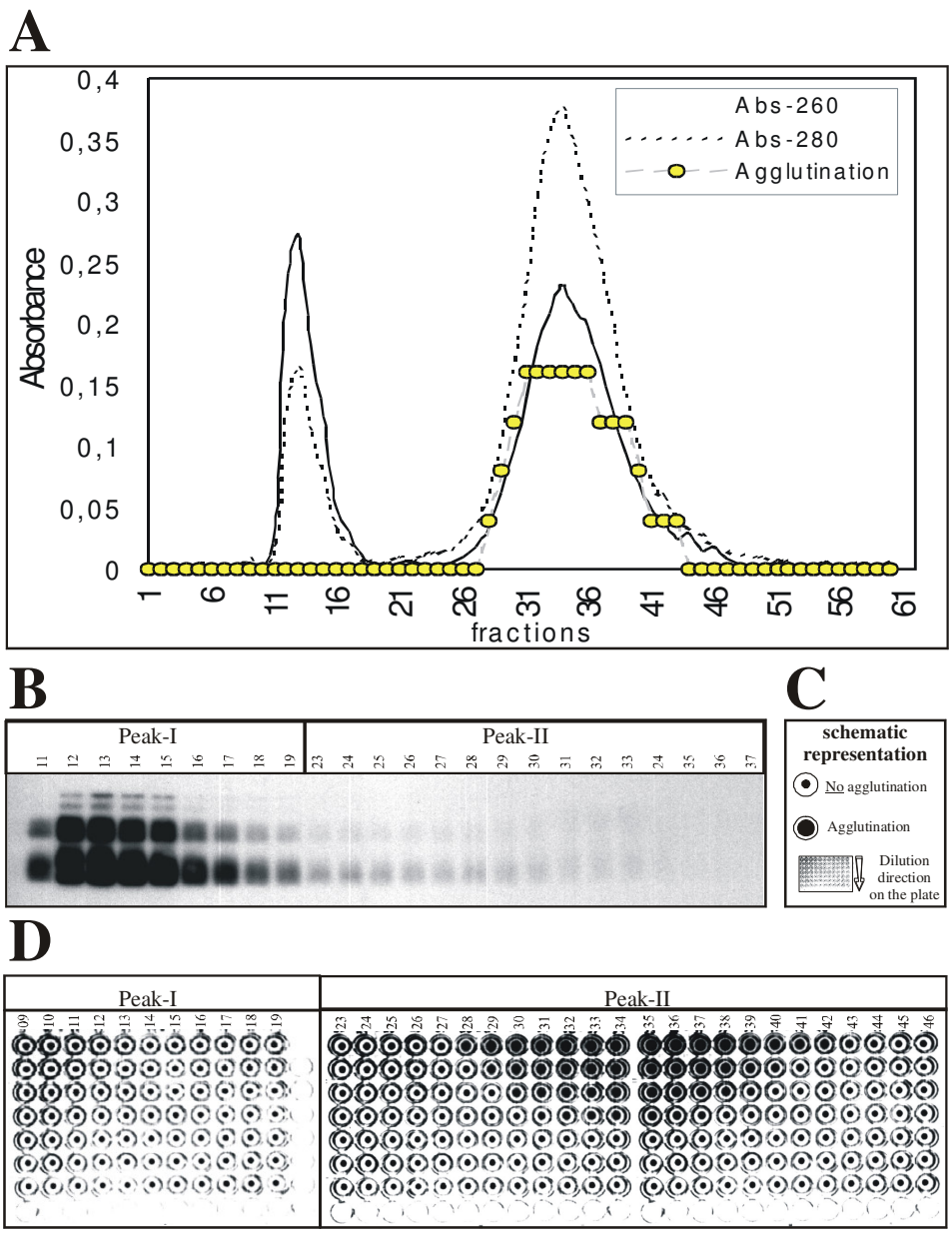

Figure 25: Hemagglutination by the fractions from BioGel agarose gel filtration experiments. (A) Chromatographic profile of fractions from BioGel agarose filtration chromatography with absorbance measurements at 260 and 280 nm. (B) Southern blot hybridization analyses of individual fractions. (C) Schematic representation of hemagglutination profiles. (D) Hemagglutination assay of chromatographic fractions.

4. 19 Analysis of circular dichroism spectra

Circular dichroism measure the optical activity of chiral molecules when circularly polarized light is transmitted through them. Biomolecules like proteins and nucleic acids, which contain monomers with chirality, are able to give rise to characteristic CD-spectra (van Holde, 1985). CD-spectra of proteins, for example, give an estimation of the presence of α-helices and β-sheets, and are, therefore, used to predict protein secondary structures (Kay, 1970; McCubbin et al., 1971). Similar studies have sought to correlate structural parameters of DNA with their CD spectra (Bloomfield et al., 2000). Another application of CD-spectrum analysis is used in studies on changes accompanying the interaction between proteins and their ligands (Zand, et al., 1971; Doyle et al., 1975). The spectral behavior of ConA during its interaction with metal ions and sugars has been previously well documented (Doyle et al., 1975). Since the spectrum profile of DNA is also well characterized, it has been worth investigating the CD-spectra of ConA-DNA-complexes.

For this purpose, the spectral characteristics of both components of the complex were determined. The CD-analyses were carried out by measuring the CD-spectrum of ConA, DNA, ConA-DNA-complex and peak I from the BioGel filtration experiment (Fig. 26 A). First, the presence and the amount of ConA in the peak I fractions was checked by SDS-polyacrylamide gel electrophoresis (Fig. 26 B). ConA-subunit, with a molecular weight of 27 kDa (Agrawal and Goldstein, 1967a) could be detected in the gel in small amounts in Peak I and in excessive amounts in peak II. The smaller band detected in peak II correspond to fragmented subunits of ConA, which was 14 kDa in size and was present in low amount in naturally isolated ConA preparations (Wang et al., 1971).

As expected, the CD-spectrum of ConA alone corresponded to that described in the literature (Zand, et al., 1971; Doyle et al., 1975), with a trough at about 220 nm and almost no optical activity for wavelengths between 250 and 300 nm. The CD-spectrum of DNA alone was typical for the B-form of DNA (Sprecher et al., 1979), and showed a maximum at 275 nm, a minimum at 245 nm and a crossover around 260 nm. Interestingly, the CD-spectrum profile of the ConA-DNA-complex showed similarities with the CD-spectra of ConA and of DNA. Moreover, the similarities were dependent on the range of wavelengths. While for wavelengths in the 250 – 300 nm range the CD-spectra of ConA-DNA-complexes overlapped completely with the CD-spectra of DNA alone, for the 190 – 250 nm range the CD-spectra of ConA-DNA-complexes approached those of the CD-spectrum of ConA alone. The CD-spectrum profile of

peak I presented very low circular optical activity, probably due to its low concentration. Nevertheless, the CD profile of peak I tended to be positioned between the CD-spectra of ConA and DNA.

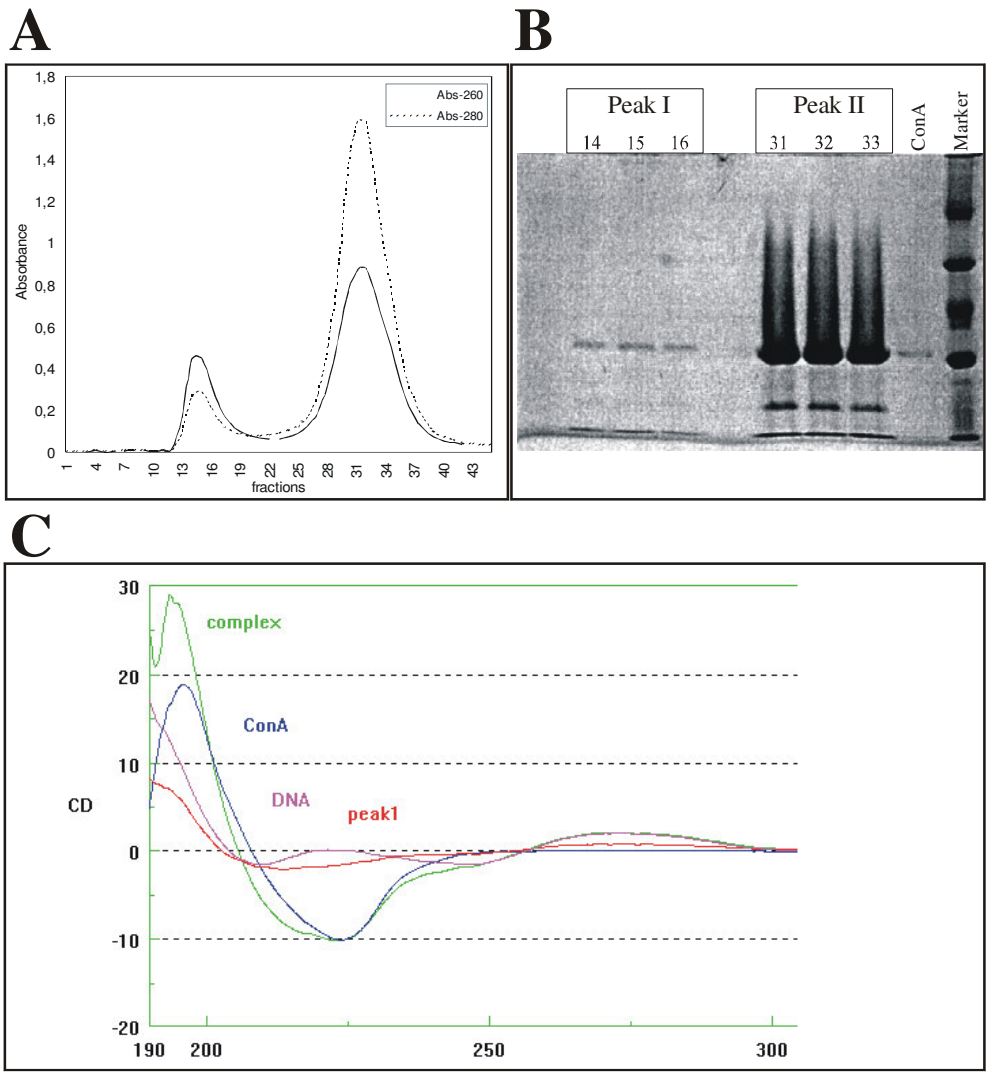

Figure 26: **Circular dichroism analyses**. (A) Chromatographic profile of fractions from the BioGel agarose filtration with absorbance measurements at 260 and 280 nm. (B) Polyacrylamide gel electrophoresis of individual fractions. (C) CD-spectra of ConA, DNA, ConA-DNA-complex and of fraction 15 from peak I.

4. 20 Uptake of ConA-DNA-complexes by cells in culture and expression of green fluorescent protein (GFP)

Lectins are not only able to bind reversibly to mono- and polysaccharides, but also to a variety of glycoconjugates, as for example those found in the cellular membranes (Noonan and Burger, 1973). The recognition of such glycoconjugates is the basis for the ability of lectins to bind to cells (Huet, 1975; Schnebli and Bächi, 1975). Lectins could, therefore, be useful tools in the targeting of biomolecules to the membrane of cells (Clarck et al., 2000). In addition to its cell binding ability, many lectins are also able to enter cells (Juliano et al., 1978; Storrie and Maurey, 1981). It was, therefore, of considerable interest to investigate whether ConA-DNA-complexes bound to and entered into cells.

Experiments presented here have shown, that during the formation of the complex between ConA and DNA, a limited amount of ConA-DNA-complex is formed and a relative large amount of ConA remains free in solution. This free ConA (peak II) can be successfully separated from the DNA-bound ConA (peak I) by BioGel agarose chromatography (Fig. 25 and 26 B). Because of the excess of ConA present in the solution during ConA-DNA-complex formation, any incubation of this solution with cells would give rise to cell agglutination due to the presence of free ConA (Fig. 18 and 19). Agglutinated cells have most of their ConA-binding sites involved in the process of agglutination itself, having only a smaller number of ConA-binding sites left for ConA-DNA-complexes to bind. For this reason, investigations on the uptake of ConA-DNA-complexes by cells had to use the peak I fractions from the BioGel agarose chromatography experiment. Fig. 27 exemplified a typical BioGel agarose gel filtration experiment.

The DNA used for the formation of ConA-DNA-complexes was the pEGFP-C1-plasmid, which carried the gene for the green fluorescent protein (GFP). For studies on the uptake of ConA-DNA-complexes by cells growing on culture plates, the detection of GFP expression was assessed by fluorescence microscopy. The appearance of green fluorescence in cells is shown in Fig. 28, 29 and 30. In general, the number of cells showing GFP-expression was relatively small in comparison to the number of cells showing no expression. Since no quantitative studies were performed, it was not possible to estimate the percent of cells expressing the GFP-gene. Mostly, green fluorescent cells were found close to cells, which were not green fluorescent (Fig. 28 B and D; Fig. 29 C, D and E; Fig. 30 B - E). Rarely, two or more green fluorescent cells were found together (Fig. 28 C; Fig. 29 B and F).

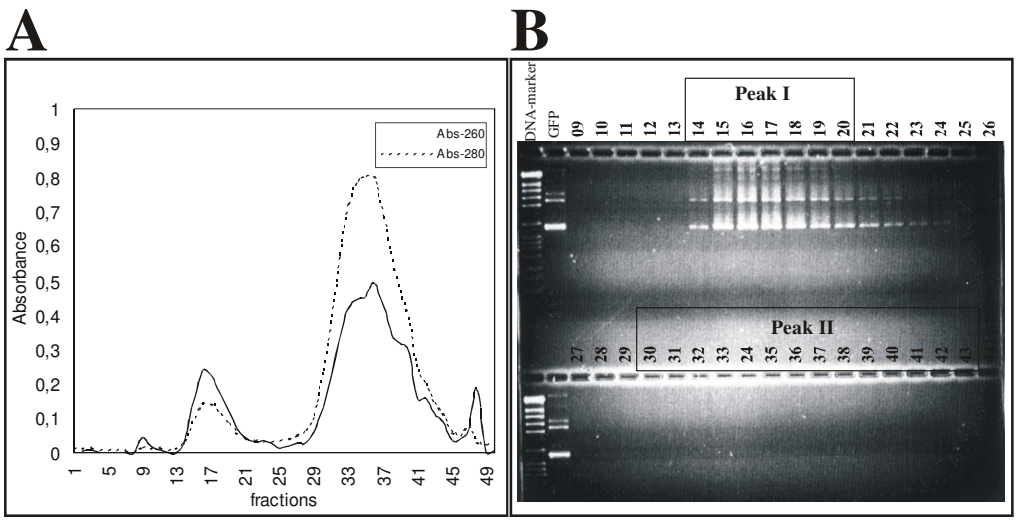

Figure 27: Example of a BioGel agarose filtration experiment of the ConA-DNA-complex, from which peak I fractions were used for incubation with cells. (A) Chromatographic profile of the fractions with absorbance measurements at 260 and 280 nm. (B) EMSA analysis of individual fractions.

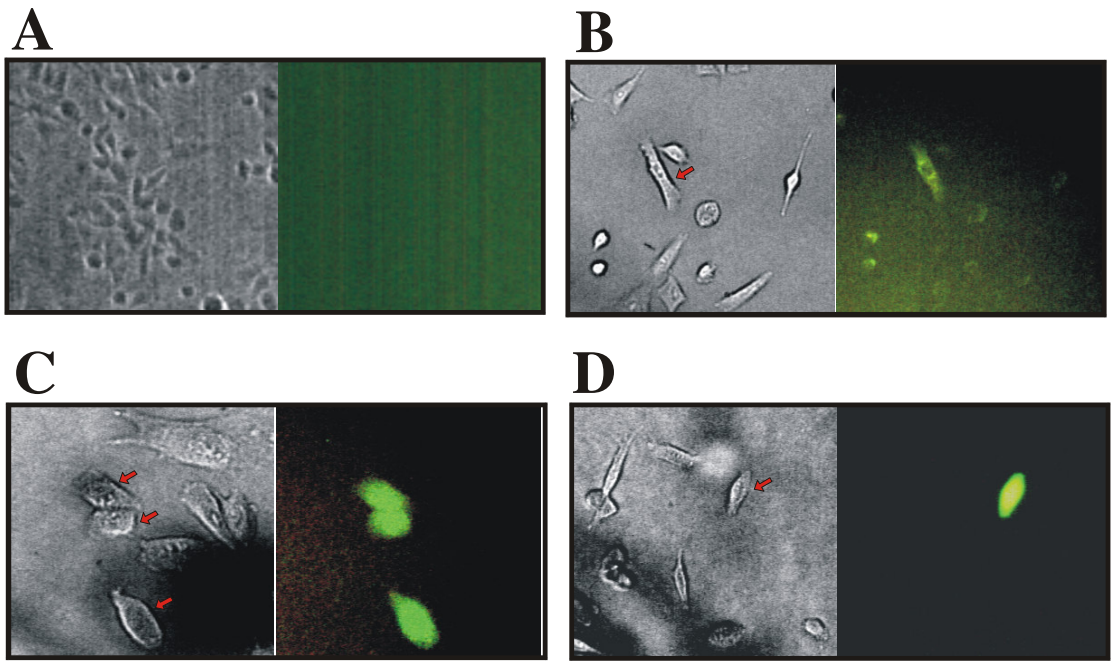

Figure 28: GFP-expression in A549-cells analyzed by fluorescence microscopy. (A) Control cells. (B), (C) and (D): Cells incubated with peak I fractions.

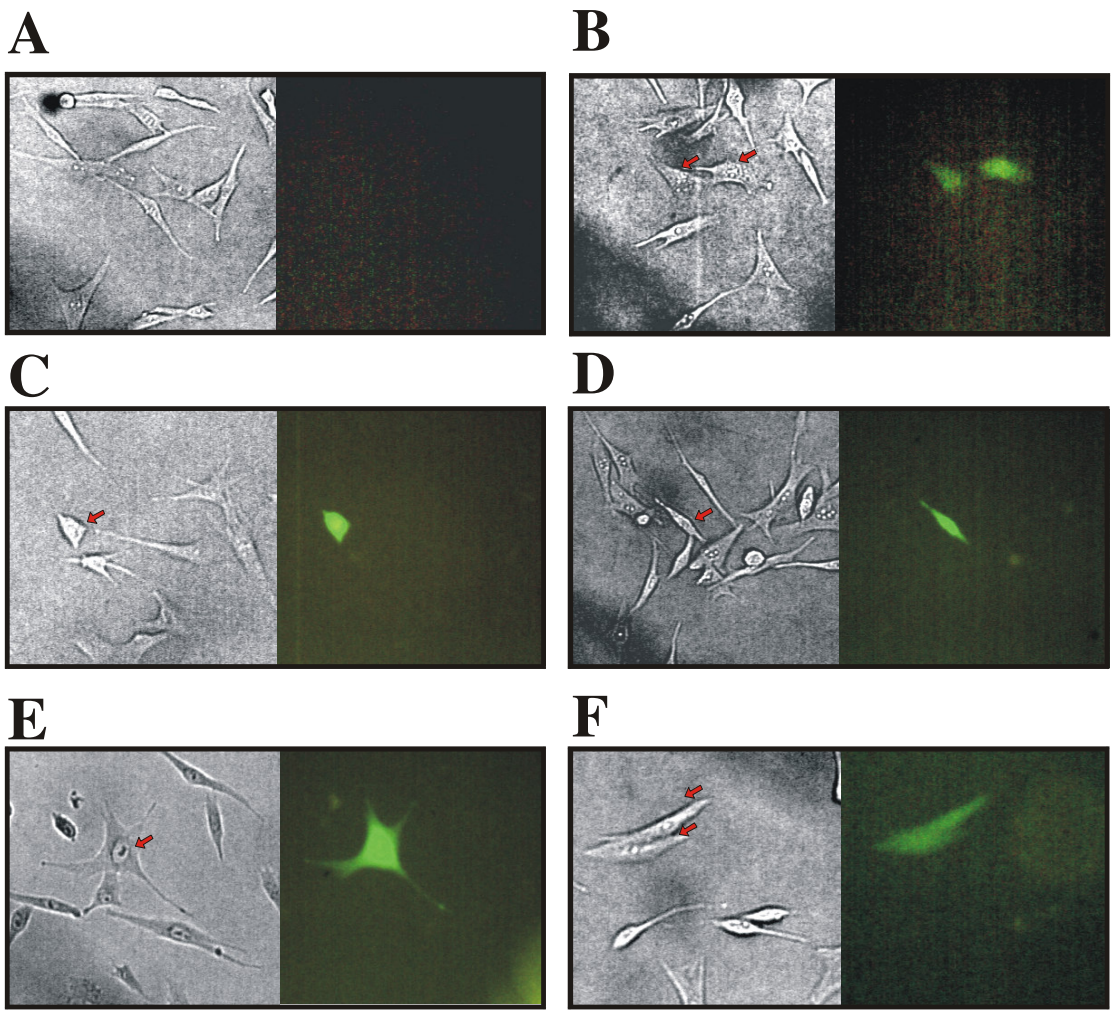

Figure 29: GFP-expression in BHK-cells analyzed by fluorescence microscopy. (A) Control cells. (B), (C), (D), (E) and (F): Cells incubated with peak I fractions.

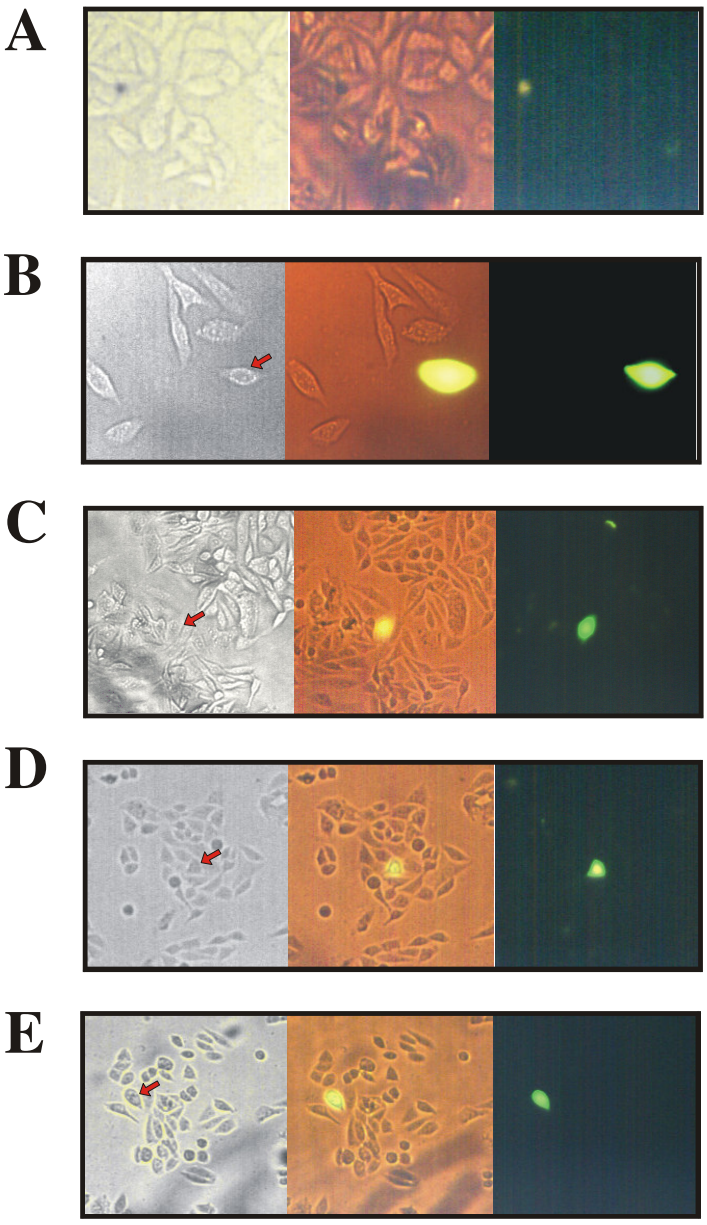

Figure 30: GFP-expression in CHO-cells analyzed by fluorescence microscopy. (A) Control cells. (B), (C), (D), (E) and (F): Cells incubated with peak I fractions.

4. 21 Formation of Lectin-DNA-complexes in other members of the Leguminosae family

The lectins of *Canavalia bonariensis* (ConBn) and *Lens culinaris* (LcA) bound to DNA and formed lectin-DNA complexes as shown by electrophoretic mobility shift assays (EMSA) (Fig. 31). As observed in ConA-DNA-complexes (Fig. 4), the DNA-shift was dependent on the lectin concentration. The DNA-shift pattern at high lectin concentrations revealed that the lectin-complexed DNA did not appeared as discrete DNA-bands. The smear-like DNA migration was more easily observed in ConBn than in LcA (Fig. 31 A and B). LcA gave arise to a similar pattern only at final lectins concentrations higher than 1 µg/µl (Fig. 31 B). Although the extent of DNA binding of these two lectins was shown to depend on concentration, none of them reached a maximum saturation point.

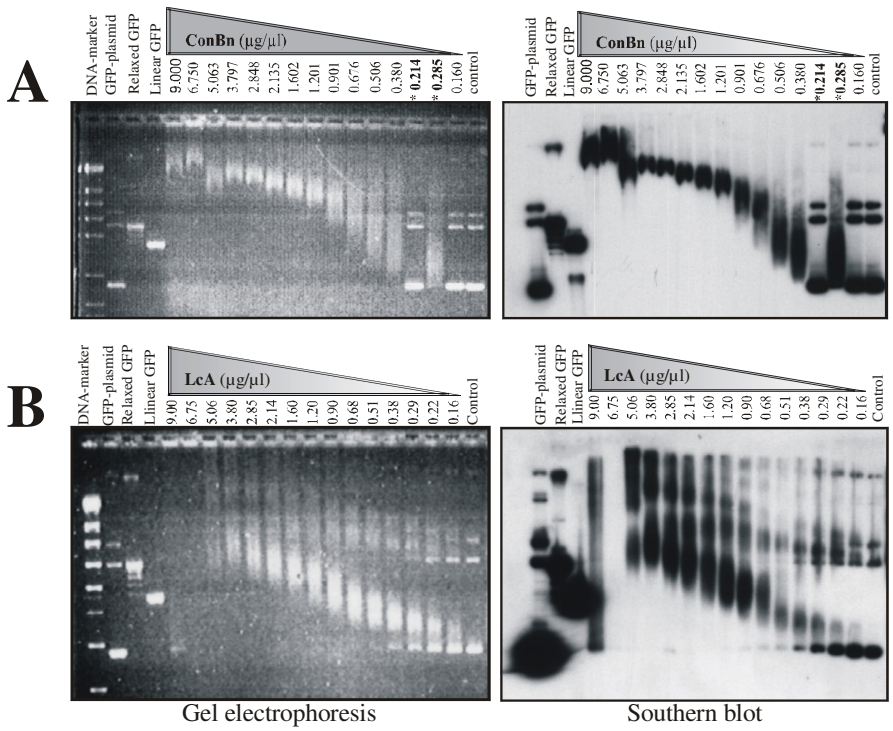

Figure 31: EMSA and Southern transfer hybridization analysis of complexes formed by incubation of pEGFP-C1 DNA and ConBn or LcA. From the left to the right: DNA-marker, GFP plasmids and lectin concentrations from 9 to 0.16 µg/µl and negative control. EMSA-analyses are represented by UV-photographs of gels (on the left) and Southern transfer hybridizations by blots (on the right). Experimental conditions as described in the legend to Fig. 4. (A) ConBn: lectin of *Canavalia bonariensis*; (B) LcA: lectin of *Lens culinaris*.

The DNA bound by the lectins of *Canavalia brasiliensis* (ConBr) and *Canavalia maritima* (ConM) revealed an electrophoretic migration pattern characterized by the simultaneous shift of all discrete bands of DNA (Fig. 32). By comparing the position of the lectin-complexed DNA with the control-DNA, it became apparent that ConBr caused more extensive shifts in DNA mobility than ConM. While ConBr-DNA-complex formation reached a maximum value, or a saturation point, between 2.85 and 3.8 µg/µl final lectin concentration (Fig. 32 A), the formation of ConM-DNA-complex showed saturation point at concentrations between 6.75 and 9 µg/µl (Fig. 32 B). Preliminary analysis of the DNA-binding ability of the lectins from *Canavalia grandiflora* (ConG) and *Dioclea violacea* (Dviol) led also to the formation of lectin-DNA-complexes (data not shown).

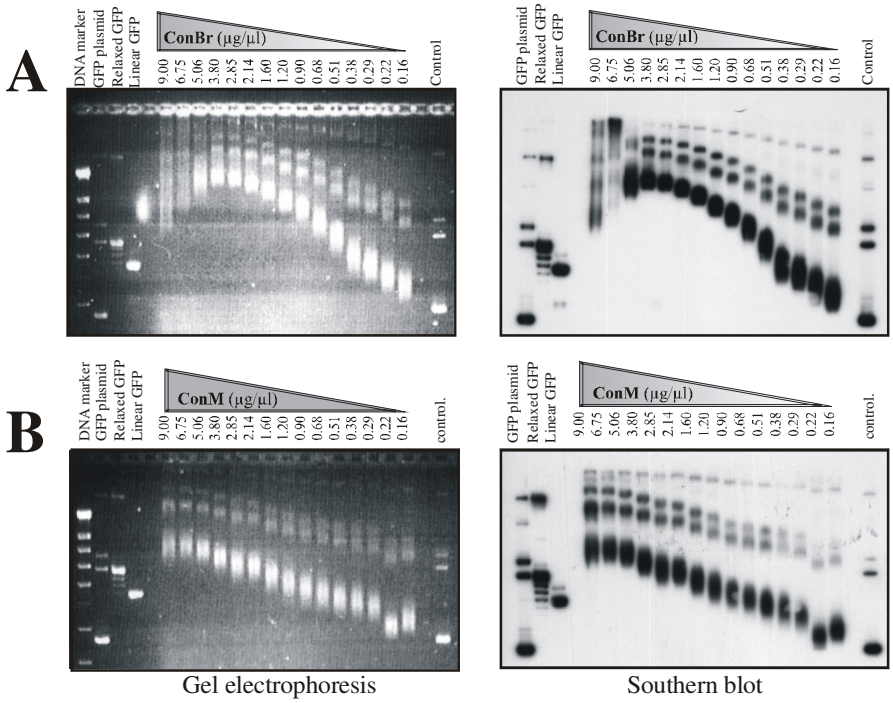

Figure 32: EMSA and Southern transfer hybridization analysis of complexes formed by incubation of pEGFP-C1 DNA and ConBr or ConM. Experimental conditions as described in the legend to Fig. 4. (A) ConBr: lectin of *Canavalia brasiliensis*; (B) ConM: lectin of *Canavalia maritima.*

The lectin of *Dioclea virgata* (Dvirg) and the isolectin of *Phaseolus vulgaris* (PHA-E) bound to DNA and formed lectin-DNA-complexes with distinct properties (Fig. 33). A direct correlation between lectin concentration and extent of DNA-shift was not observed for Dvirg-DNA-complexes. Based on the position of the DNA-shifts, the saturation point of Dvirg-binding

to DNA was reached at lectin concentrations between 0.9 and 2.14 µg/µl. Interestingly, Dvirg concentrations higher than 2.14 µg/µl resulted in a lesser DNA-shift. A complete absence of DNA-shifts was observed at Dvirg concentrations between 6.75 and 9 µg/µl (Fig. 33 A). The isolectin of *Phaseolus vulgaris* (PHA-E) bound to DNA in a concentration dependent manner (Fig. 33 B) but caused a much weaker DNA-shift compared to other lectins studied (Fig. 4 and 31). Although the majority of lectins investigated bound equally well to oligomeric, nicked and supercoiled DNA, PHA-E preferred supercoiled DNA. Since the nicked plasmid DNA was not shifted by this lectin, the interaction between PHA-E and DNA seemed to depend rather on plasmid conformation (Fig. 33 B). Furthermore, PHA-E was not able to bind to linearized plasmid either (data not shown).

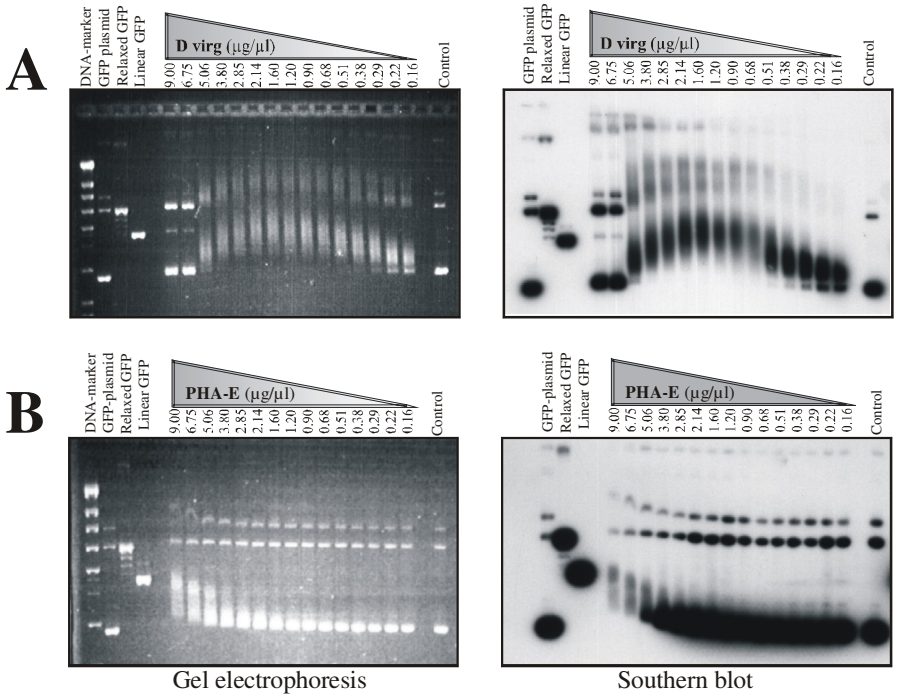

Figure 33: EMSA and Southern transfer hybridization analysis of complexes formed by incubation of pEGFP-C1 DNA and Dvirg or PHA-E. Experimental conditions as described in the legend to Fig. 4. (A) Dvirg: lectin of *Dioclea virgata*; (B) PHA-E: isolectin of *Phaseolus vulgaris* (erythroagglutinin).

Leguminous lectins from *Vicia villosa* (VVLB4), *Arachis hypogea* (PNA), *Glicine max* (SBA) and the isolectin from *Phaseolus vulgaris* (PHA-L) were not able to form complexes with DNA and did not give arise to shifts in DNA migration (data not shown).

4. 22 Partial inhibition of the formation of lectin-DNA-complexes by specific sugars

Once the conditions for the sugar inhibition assays had been established for ConA, three other lectins were tested using the same experimental procedure. The DNA-binding ability of ConBn was not influenced by the presence of specific sugars (Fig. 34 A). Complex formation of DNA with ConBr and ConM was partially inhibited by methyl-α-D-glucopyranoside or methyl-α-D-mannopyranoside (Fig. 34 B and C). Once again, galactose did not influence the extent of the DNA shift, hence had no effect on the formation of lectin-DNA complexes.

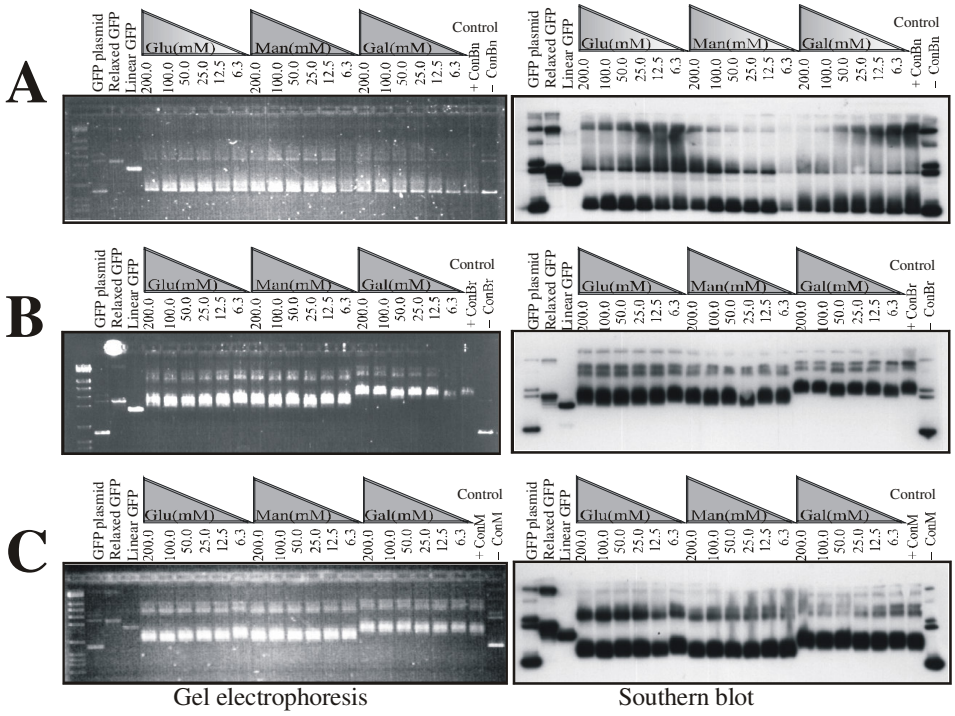

Figure 34: **Partial inhibition of lectin-DNA-complex formation by specific sugars**. The DNA-complex formation with the lectin of *Canavalia bonariensis* was not greatly influenced by the presence of sugars (A). A reduction in the shift of DNA was achieved by pre-incubating the lectin of *Canavalia brasiliensis* (B) or the lectin of *Canavalia maritima* (C) with methyl-α-D-glucopyranoside or methyl-α-D-mannopyranoside. The presence of galactose did not influence lectin-DNA-complex formation. (see also Appendix, Table 01).

4. 23 Inhibition of the formation of lectin-DNA-complexes by adenosine-5'-triphosphate (ATP)

The DNA binding ability of ConBn, ConBr and ConM was efficiently inhibited by ATP (Fig. 35). The efficiency of inhibition by ATP was much higher then the partial inhibition caused by the lectin-specific sugars (Fig. 34). For all lectins tested, ATP-concentrations as low as 5 mM were already sufficient to completely obliterate lectin-DNA-complex formation (Fig. 35).

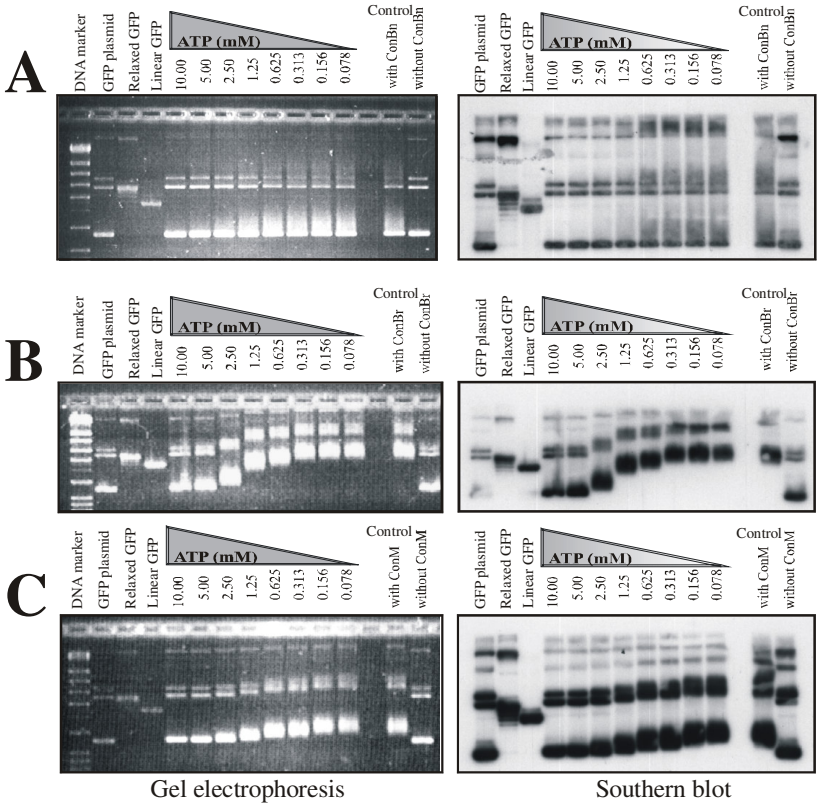

Figure 35: Adenosine-5'-triphosphate (ATP) inhibits lectin-DNA-complex formation. The inhibition of the lectin-DNA-complex formation was achieved by pre-incubating the lectins with ATP at different concentrations. (A) ConBn: lectin of *Canavalia bonariensis*; (B) ConBr: lectin of *Canavalia brasiliensis*; (C) ConM: lectin of *Canavalia maritima*.

5. DISCUSSION

5. 1 Lectin-DNA-complex formation

The present study revealed the interaction of several leguminous lectins with DNA and the formation of lectin-DNA-complexes. Upon lectin binding, the electrophoretic migration of the bound DNA decreased, causing a DNA-shift that could be detected by gel electrophoresis. Under the experimental conditions applied, 9 out of a total of 13 leguminous lectins showed DNA binding activity. The lectins ConA, ConBr, ConBn, ConM, ConG, Dviol, Dvirg, LcA and PHA-E were able to bind DNA (Fig. 04, 31, 32 and 33). The pattern of DNA-shift was dependent on the lectin used. ConBr- and ConM-binding effected a DNA-shift with discrete bands (Fig. 32). The other lectins caused a smear-like DNA-shift pattern (Fig. 04, 31, 33). The lectins PHA-L, PNA, SBA and VVLB4 did not form lectin-DNA-complexes and were not able to cause DNA-shift (data not shown).

The concentration-dependent modus of binding and the formation of smear-like migration patterns (ConA-, ConBn- and LcA-complexes) might indicate a cumulative effect of binding that caused a progressive saturation of DNA by lectins (Kabanov et al., 1998). A saturation point of maximal binding was difficult to estimate, because at very high lectin concentrations the lectin-DNA-complexes barely entered the agarose gels (Fig. 04 and 31). The utilization of DNAs with smaller size and of gels with lower agarose concentrations might be more adequate to estimate the saturation point for lectin-DNA-complexes (Fairall et al., 2000; Laniel et al., 2001). The multimeric nature of these lectins might explain the smear-like pattern of DNA-migration and the cooperative-like mode of binding. Considering that each subunit was able to interact with DNA, a multimeric lectin (dimeric or tetrameric) would offer more than one site for DNA-binding. According to this hypothesis, a tetrameric lectin would bind more DNA, causing more extensive DNA-shift, than a dimeric lectin. The DNA-shifts caused by the tetrameric lectins ConA and ConBn (Fig. 04 and Fig. 31 A), in comparison to the DNA-shifts caused by the dimeric lectin LcA (Fig. 31 B), seemed to confirm this prediction. Theoretically, the multimeric structure of the lectin might allow two levels of binding: (i) the lectin would bind to two segments of the same plasmid DNA or (ii) the lectin would bind independently to two plasmid DNA molecules. In the first scenario, an increase of lectin concentration would cause a ”condensation“ of each DNA-molecule. This effect might be responsible for the independent

DNA-shifts caused by the lectins ConBr and ConM (Fig. 32). The second model, the lectin-DNA-binding would cause a "cross-linking" of two independent plasmid DNA molecules. In this case, lectin-complexed DNAs would randomly aggregate with each other and no discrete DNA-bands in electrophoresis gels would be observed. Since during complex formation many lectin molecules could bind to the same plasmid molecule, a combination of the two suggested binding-scenarios might also be possible. The formation of lectin-DNA-complexes with short DNA-fragments, which allow the binding of only a limited number of lectins per DNA-molecule, might answer the question of the multiplicity of lectin binding to DNA (Kabanov et al., 1998).

The three naturally occurring forms of the pEGFP-C1-plasmid, supercoiled, nicked and oligomeric, were used as DNA-partners in the lectin-DNA binding reactions. The supercoiled and oligomeric plasmid forms, which have negative supercoiling, are topologically distinct from the nicked form which is topologically equivalent to relaxed plasmid (Bauer, 1978). The majority of the lectins able to form complexes with pEGFP-C1-plasmids were not able to distinguish different plasmid conformations (Fig. 04, 31, 32 and 33 A). The isolectin of *Phaseolus vulgaris* (PHA-E) was the only lectin which showed preferential binding to supercoiled DNA (Fig. 33 B). While nicked plasmids were almost not shifted by this lectin, supercoiled and oligomeric plasmids were. To confirm the preference of PHA-E for supercoiled DNA, binding reactions were performed using EcoRI-linearized plasmids. No DNA-shift of PHA-E with linearized plasmid DNA could be observed (data not shown).

Since DNA with negative supercoils are underwound, the number of base pairs per turn is lower than in relaxed DNA. Therefore, in supercoiled DNA the bases are slightly more exposed for the recognition by proteins (Bloomfield et al., 2000). The different exposure of the DNA-bases plays a role particularly in sequence-specific recognition processes (Church et al., 1977). On the other hand, proteins like topoisomerases, HMG- and histone-like proteins, which are known to recognize different DNA-structures, bind preferentially to bent, kinked, or wound DNAs (Vogel and Singer, 1975; Stros and Reich, 1998). Since the mechanism of lectin-binding to DNA is not known, the reasons for preferential binding of PHA-E to supercoiled DNA remains to be clarified.

5. 2 Characterization of ConA-DNA-complexes

5. 2. 1 Kinetics of ConA-DNA-complex formation

Experiments which followed the formation of ConA-DNA-complexes over time, showed that the binding reaction was very fast. Already one minute after incubation, all DNA available for the reaction was shifted and discrete DNA-bands were no longer detected (Fig. 10 B). The ConA-DNA binding reaction continued and an increase in DNA-shift could be observed up to one hour after incubation (Fig.10 A). In the subsequent hours, no substantial increase in DNA-shift was observed (data not show). After the first three minutes of incubation, ConA-DNA-complexes migrated in a smooth smear-like pattern, whereas after one hour the migration was rather like a discrete DNA-band. These two patterns of DNA-migration indicated that ConA-DNA-complexes became more homogeneous in size with time. This observation supported the notion of an accumulative mode of DNA binding to ConA (Kabanov et al., 1998).

5. 2. 2. No evidence for sequence specificity

ConA shifted supercoiled and nicked closed pEGFP-C1-plasmid DNAs equally well and efficiently as the linearized plasmid and the subfragments of it (Fig. 04 and 09). The binding of ConA to a mixture of DNA-fragments from diverse sources (Fig. 09 C) indicated that DNA recognition was probably not sequence-specific. Nevertheless, the possibility that CG- or AT-rich sequences were preferentially bound by ConA remained to be tested. This could be e.g. achieved by the utilization of short DNA-fragments with know CG- and AT-contents as DNA-binding partners for the reaction with ConA. The DNA-shift pattern shown in Fig. 09 C reinforced the notion of “multiplicity of binding” and of “cross-linking” effects, discussed in section 5. 1. The smear-like DNA-shift of all ConA-bound fragments indicated that the absence of discrete DNA-band shifts was not only a matter of the size of the ConA-complexed DNA. Even the smallest fragment (300 bp) was not shifted as a discrete DNA-band.

5. 2. 3. The size of ConA-DNA-complexes

The constant charge/mass ratio of nucleic acids is the major factor that permits the electrophoretic separation of different sizes of naked DNA in agarose gels (Fairrall et al., 2000; Laniel et al., 2001). DNA’s conformation is another factor that influences DNA-migration in agarose gels. Both parameters might change considerably when DNA is complexed with proteins. In protein-DNA-complexes, the total charge and the size of the complexes are highly dependent on the nature of the protein component (Bloomfield et al., 2000). Additionally, DNA-binding proteins might cause changes in the shape of single DNA-molecules, or even induce

aggregation of complexed DNAs (Kabanov et al., 1998). Neither the charge/mass ratio nor the size of lectin-DNA-complexes has been precisely determined.

In non-specific protein-DNA-complexes, the binding protein-DNA occurs randomly, giving arise to complexes within a relatively broad range of sizes. The characterization of the formed protein-DNA-complexes can be facilitated when the complexes are homogeneous in size (Kabanov et al., 1998). Normally, an increase in homogeneity is obtained by a variation of the relative concentration of protein and DNA until an optimal molar ratio between protein and DNA is found. Conditions, where an excessive amount of protein is incubated with low amounts of DNA, may provide a saturation point at maximal binding. Under optimal conditions, proteins occupy all available sites on the DNA, and the excess of non-bound proteins remains free in solution.

An experiment was designed to obtain ConA-DNA-complexes more homogeneous in size (section 4. 8). For technical reasons, the maximal final ConA-concentration applied in these experiments did not exceed 12 µg/µl. The preparation of stock solutions of ConA at concentrations higher than 15 µg/µl in low ionic strength buffers caused protein aggregation or even precipitation. Under standard experimental conditions for lectin-DNA-complex formation (9 – 0.16 µg/µl ConA and 6 ng/µl DNA), a saturation of ConA-binding to DNA could not be reached (Fig. 04). Since a further increase in ConA-concentration was limited by protein solubility, variation in DNA-concentration was preferred. During the binding reactions described here, the ConA-concentration was kept constant (9µg/µl), and the DNA-concentration was varied (0.391 – 200 ng/µl). The results shown in Fig. 11 indicated that the ConA-DNA-complexes were too big to enter the gel. An excess of free ConA could be observed in EtdBr-stained gels (Fig. 11 A). The migration of ConA-DNA-complexes in samples formed at high DNA-concentration indicated that increased DNA-concentrations led to the formation of ConA-DNA-complexes with rather heterogeneous sizes.

5. 2. 4. Separation of ConA-DNA-complexes and free ConA

A chromatographic separation of ConA-DNA-complexes (peak I) from the uncomplexed, free ConA (peak II) was accomplished by gel filtration in agarose BioGel columns (Fig. 24, 25, 26 and 27). A number of exploratory gel filtration experiments was carried out to find the optimal conditions for the separation of peak-I and peak-II fractions, which were subsequently analyzed by EMSAs. The DNA-shift patterns of fractions from peak I were used as parameter to estimate the stability of the eluted ConA-DNA-complexes. This estimation was particularly important when fractions of peak I were prepared for subsequent use in other experiments (sections 4. 18, 4. 19 and 4. 20). ConA-DNA-complexes from peak I fractions were stable for

more than 24 h at room temperature (data not show). The abundance of DNA-complexed ConA in peak I and free-ConA in peak II was dependent on the ConA/DNA-ratio and on the buffer used during complex formation. In general, the vast majority of ConA applied to form complexes with DNA was detected as free ConA in peak II fractions (Fig. 26 B). A comparison of the amount of DNA-complexed ConA (peak I fractions) with the amount of free ConA (peak II fractions) might provide an estimate of the binding constant of ConA-DNA-binding. These experiments to determine the ConA-DNA binding constant was not carried out so far, because difficulties in finding a saturation point of maximal binding complicated further quantitative analyses. Moreover, the chromatographic procedure itself might affect the binding equilibrium (association/dissociation) of the binding partners.

5. 2. 5. Circular dichroism analyses of ConA-DNA-complexes

Circular dichroism analyses (CD-spectra) of ConA performed in the early 1970s had already indicated the β-sheet rich secondary structure characteristic of this lectin as well as the absence of α-helix (Kay, 1970; McCubbin et al., 1971). X-ray structure analyses of ConA confirmed the secondary structure predicted by CD-spectra. CD-analyses were also applied to predict changes in the conformation of ConA upon metal ion and sugar binding (Doyle et al., 1975). The pH dependence of the conformational changes of ConA was also approached by CD-spectra analyses (Zand, et al., 1971). Since the spectral behavior of ConA was well documented, it was worth investigating the CD-spectra of ConA-DNA-complexes. Circular dichroism analyses of ConA-DNA-complexes showed a spectrum profile that corresponded to the sum of the spectra of ConA and DNA (McCubbin et al., 1971; Sprecher et al., 1979) (Fig. 26 C). In general, the CD-profile of ConA-DNA-complexes exhibited closer similarities with the CD-profile of ConA alone. This result could be explained by the observation that, after complex formation, most of the ConA remained as free-ConA in solution (peak II in Fig. 24, 25 and 26). The CD-spectrum of peak I, which corresponded to ConA-DNA-complexes without excess of free-ConA, showed a pattern intermediate between those of ConA and DNA. Computer calculations indicated that the CD-spectra of peak I could be closely approximated by a linear combination of the spectra of the unbound components. This result implied that no conformational changes occurred upon binding of ConA to DNA. But these measurements were not conclusive so far, because the detection of different conformations by CD-spectrum analyses was representative only when a series of spectra at different temperatures were compared. Nevertheless, the CD-spectrum analysis of peak I unequivocally documented the presence of ConA in peak I (McCubbin et al., 1971).

5. 2. 6. Recovery of intact DNA from ConA-DNA-complexes

Experiments, in which the protein content of the ConA-DNA-complex was removed by proteinaseK/SDS treatment and phenol-chloroform extraction, indicated that the lectin-DNA-binding was reversible and did not substantially alter the DNA (Fig. 13). The recovery of DNA from ConA-DNA-complexes was more quantitative for linearized plasmid DNAs than for supercoiled closed circular plasmids (see Fig. 13, samples with 9µg/µl ConA). It was possible that these differences in DNA recovery efficiency reflected the accessibility of the DNA-bounded ConA for enzymatic degradation by proteinaseK. The spatial arrangement of ConA-molecules in ConA-DNA-complexes derived from linearized- as compared to closed circular-plasmid was probably distinct. According to this notion, ConA-molecules that bound to DNAs with different topologies would be differentially "shielded" from proteolytic attack. On the other hand, it was also possible that the accessibility of DNA in ConA-DNA-complexes was altered in comparison to naked DNA. Was the DNA in ConA-DNA-complexes "protected" against nuclease attack? A treatment of ConA-DNA-complexes with nucleases and an analysis of the generated DNA-fragments might answer this question.

5. 2. 7. The ionic nature of the ConA-DNA binding

Ionic strength is one of the most important factors affecting non-specific protein-DNA-interactions (Schwabe, 1997; Bloomfield et al., 2000). Cations can strongly bind to DNA, because of the polyanionic nature of this nucleic acid. In buffer solutions, DNA molecules are interacting with a number of positively charged ions (Xu and Brewer, 1997; Li et al., 1998). Upon binding to positively charged macromolecules, ions are released from the DNA into the solvent. This release leads to an increase in entropy (Honig and Nicholls, 1995). If a solution already has a high concentration of ions, any ionic interaction between the DNA and a positively charge macromolecule will not be favored (Bloomfield et al., 2000). Therefore, the addition of salt alters ionic interactions of proteins with DNA. Analyses of the influence of ionic strength on the formation of ConA-DNA-complex revealed a strong correlation, indicating that the interaction between ConA and DNA was likely to be of ionic nature (Fig. 17). An excess of salt had a negative effect on complex formation, especially when the ions were present during the binding reaction (Fig. 17 C). Only high concentrations of salt could disrupt already existing ConA-DNA-complexes (Fig. 17 B). In the presence of salt (NaCl) at physiological concentrations (150 mM), ConA-DNA-complexes did not form. Nevertheless, already formed ConA-DNA-complexes remained stable when salt was added at a final concentration of 150mM (Fig. 17 B). The stability of ConA-DNA-complexes at physiological NaCl concentration was particularly important for the incubation of these complexes with cells in culture (section 4. 20).

ConA is stable at 37°C at a broad range of NaCl-concentrations. The two major factors that influence ConA-binding to specific sugars are ConA-conformation and the occupancy of the two metal-binding sites close to the sugar-binding site. The recognition and binding of ConA to glucose/mannose is mediated by hydrogen bonding between the protein and the carbohydrate. In the presence of metal ions (Ca^{2+} and Mn^{2+}), ConA is able to bind to its specific sugars at low and at high salt concentrations (Senear and Teller, 1981b). The binding occurred even at 1M NaCl-concentration. Therefore, the impact of ionic strength on the ConA-DNA- and ConA-carbohydrate-interactions can be quite different.

5. 2. 8. The effect of pH on ConA-DNA-complex formation

The pH-dependent dimer/tetramer equilibrium has been reported for many lectins of the *Diocleinae* tribe (Dam et al., 1998; Ramos et al., 2000). In the case of ConA, this pH-dependent equilibrium and the abundance of dimeric and tetrameric forms at a broad range of pH values have been well documented (McKenzie and Sawyer, 1973; Senear and Teller, 1981a). At low pH values, ConA exists as a dimer, at pH values over 7.0 ConA exists as a tetramer. At pH values between 5.0 and 7.0, a mixture of ConA dimers and tetramers predominates. To check whether different quaternary structures of ConA would show different binding affinities for DNA, binding reactions were performed at pH 5.0, 6.0 and 7.5 (Fig. 12). A direct correlation between pH and the efficiency of ConA-DNA-complex formation could be observed, indicating a possible correlation between ConA's quaternary structure and its affinity for DNA. Obviously, it was necessary to consider the consequences of lower pH-values on both binding partners. A considerable number of phosphate-groups of the DNA-backbone was protonated at low pH-values. Protonated phosphates are no longer negatively charged and, therefore, not available for ionic interactions with positively charged amino acid residues of the protein (Bloomfield et al., 2000). A pH-dependent displacement of metal ions, which are coordinated with the phosphate or ribose residues, might also change local charges on the DNA (Izatt et al., 1971). The pH-dependent efficiency of ConA-DNA-complex formation confirms, to a certain extend, the ionic character of the ConA-DNA-interaction. A better estimation of the influence of quaternary structure on ConA-DNA-binding might be obtained, when binding reactions are carried out under optimal conditions for ConA-DNA-complex formation. In order to compare the efficiency of ConA-DNA-complex formation using dimers or tetramers of ConA, binding reactions should be carried out at pH 7.5. A commercially available ConA, which is covalently linked to a Succinyl-group, does not form tetramers even at high of pH-values (Gunther et al., 1973). This Succinyl-ConA could be used for ConA-DNA-complex formation at pH 7.5.

The results presented in Fig. 17 indicated that the binding of ConA to DNA was likely to be ionic. But this observation did not implicate necessarily that ConA was positively charged and was interacting with the negative charges of the DNA. The isoeletric point (pI) of ConA was reported to be at 7.1 (Agrawal and Goldstein, 1967a; Zand et al., 1971). Variations of this pI-value might vary dependent on ConA metallization levels. At pH-values lower than 7.1, ConA possesses a positive net charge, and at pH-values higher than 7.1, the net charge of ConA is negative. If the binding of ConA to DNA were dependent on the net charge of ConA, one would expect the opposite result of that shown in Fig. 12. Therefore, the polyanionic character of DNA did not necessarily explain the affinity of ConA to DNA. Examples of ionic interactions between ConA and polyanions were reported for sulfated glycosaminoglycans (chondroitin sulfate, heparin sulfate and heparin) (Moris and Chan, 1978; Monge et al., 1989). In these cases, the binding was only detected at low pH and low ionic strength (Toda et al., 1981). The interaction between heparin and ConA was completely inhibited by a salt concentration of 0.5 M and was substantially enhanced by the presence of Ca^{2+} and Mn^{2+} (Monge et al., 1989). The binding activities of ConA to sulfated glycosaminoglycans were considered to be the result of nonspecific electrostatic interactions. Comparing the interactions ConA-heparin and ConA-DNA many differences were apparent. Although both interactions presented ionic character, the pH- and metal-dependencies were exactly contrary. ConA bound better to DNA at high pH and in the absence of metal ions (Fig. 12 and 14).

The quaternary association pattern of ConA, like that of many other lectins, can be understood as a tetramer existing due to the interaction of two dimers (Pabru et al., 1999). The interfaces of the dimers, which interact with each other, are mostly composed of β–sheet strands (Srinivasan et al., 1996). Amino acid residues involved in the tetramer stabilization are localized in the contact-surfaces between the two dimers. In ConA-tetramers, these amino acid residues are located inside the tetramer and, hence, not available for interactions with outside ligands (Pabru et al., 1999). Therefore, these amino acid residues cannot be directly involved in the binding of ConA to DNA at pH 7.5: Interesting questions arise. Would a tetrameric ConA, which is bound to DNA in a ConA-DNA-complex, still be able to dissociate into dimers by a decrease in pH? Does the complex formation with DNA alter the equilibrium dimer/tetramer of ConA-molecules? An analysis of the behavior of preformed ConA-DNA-complexes during a gradual change of pH might be an appropriate experimental approach to answer these questions. For example, upon the formation of ConA-DNA-complexes at pH 7.5, the pH could be lowered and the complexes analyzed.

5. 2. 9. The effect of metal ions on ConA-DNA-complex formation

Since conformational state plays a major role in the interaction between biomolecules, we have paid special attention on factors that might influence ConA conformation. The occupancy of ConA subunits by metal ions is the major factor influencing the conformation of ConA (Becker et al., 1975). When the S1-site is occupied by Mn^{2+} and the S2-site by Ca^{2+}, the sugar-binding site of ConA exhibits full capacity for carbohydrate binding, and ConA is then in a conformation designated "locked" (Karlstam, 1973; Stark and Sherry, 1979). This "locked" conformation differs substantially from the conformation of metal-free ConA, which is denominated "unlocked" (Brown et al., 1977; Sherry et al., 1978; Hardman et al., 1982; Brewer et al., 1983a; Bouckaert et al., 2000). The unlocked conformation binds metal ions and carbohydrates only weakly (Shoram et al., 1978; Brown et al., 1982; Brewer et al, 1983b; Bouckaert et al., 1995). Investigations on DNA-binding activity (Fig. 04 and 09) have been performed in 10mM Tris-HCl buffer pH7.5 in the absence of metal ions. To analyze the influence of changes in conformation on the binding affinity of ConA for DNA, the binding of ConA to DNA was performed after this lectin had been pre-treated with an excess of metal ions Ca^{2+}, Mn^{2+} or Mg^{2+}. These conditions would favor the formation of metallized, locked ConA (Brown et al., 1977; Stark and Sherry, 1979). The results indicate that the presence of metal ions decreases the affinity of ConA for DNA (Fig. 14). It appears therefore plausible to assume that the fully metallized ConA in the locked conformation does not bind to DNA. Binding reactions in the presence of EDTA indicate that partially or the non-metallized ConA forms interact more readily with DNA. Whether poorly metallized ConA-molecules, which are able to bind to DNA, are in the "locked" or "unlocked" conformations remains to be determined. However, it should be mentioned, that metal ions Ca^{2+}, Mn^{2+} and Mg^{2+} also bind to DNA molecules and might influence double helix surface charges, conformation and/or stability (Eichhorn, 1981; Sigel, 1993). The major site of interaction of these metal ions is the phosphate group, but Mn^{2+} ions can also bind to the nucleotide bases (Bloomfield et al., 2000).

The analyses of the ConA-DNA-complex formation in the presence of EDTA demonstrate the impact of divalent metal ions on ConA's binding affinity for DNA. A sequestration of metal ions by EDTA causes remarkable changes in the formation of ConA-DNA-complexes (Fig. 15). In the presence of 1 mM EDTA, the complex is more stable than in the absence of EDTA (Fig. 15 A). Moreover, an increase of complex formation has been observed for EDTA concentrations between 3.75 and 0.0293 mM (Fig. 15 B). These results imply that a reduction of the metal content in ConA-molecules contributes to the binding of ConA to DNA. Most likely, the affinity of metal-free ConA for DNA is higher than its partially

metallized counter parts. The question of whether the metal-free, unlocked ConA conformation binds better to DNA, remains to be investigated. Interestingly, a decrease in the binding of ConA to DNA has been observed for EDTA concentrations higher than 7.5 mM. One cannot exclude the possibility, that very high concentrations of EDTA also cause changes in DNA conformation by sequestering bound metal ions (Xu and Bremer, 1997; Li et al., 1998; Chin and Dickerson, 2000). An environment poor in metal ions seems to favor the formation of ConA-DNA-complexes. The results presented in Fig. 15 B gave arise to two intriguing questions. If poorly metallized ConA bound DNA indeed better, why would an excess of EDTA then cause a reduction in ConA-DNA-complex formation? Which form of ConA binds better to DNA, the partially metallized or the demetallized form? To answer these questions, ConA was demetallized by extensive dialysis against TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.5), and this buffer was also used for complex formation with DNA (section 4. 12. 2). Demetallized ConA bound to DNA and formed ConA-DNA-complexes; their migration was retarded in gel electrophoresis (Fig. 16 A, right). When demetallized ConA was remetallized, its ability to bind DNA and to form complexes was lost (Fig. 16 A, left). ConA-DNA-complexes, formed with demetallized ConA, were stable at room temperature for hours (data not shown). Hence, the addition of metal ions to established ConA-DNA-complexes did not disrupt the complexes. Upon the addition of metal ions to preformed ConA-DNA-complexes, a co-precipitation of the complexes was observed (Fig. 16 C). It is know that demetallized ConA has a markedly reduced affinity for sugars (Brow et al., 1982; Brewer et al., 1983b). Accordingly, demetallized ConA does not bind to Sephadex column (Karlstam, 1973). ConA-DNA-complexes, formed from demetallized ConA were not able to bind to Sephadex either (Fig. 23). The results presented in sections 4. 11 and 4. 12 confirmed the notion that demetallization of ConA increased its capacity to bind DNA. Thus, the observed reduction of ConA-DNA-complex formation at high EDTA-concentrations (Fig. 15 B) is probably due to an influence of this chelating agent on DNA rather than on ConA.

5. 3 Inhibition of lectin-DNA-complex formation

5. 3. 1. The partial inhibition caused by methyl-α-D-glucopyranoside or methyl-α-D-mannopyranoside

The identification of an inhibitor for the binding of ConA to DNA would give a hint at the location of the binding site in this lectin (Becker et al., 1971; Becker et al., 1975). Therefore, it was investigated whether the occupancy of the sugar-binding site compromised binding to DNA. The sugars methyl-α-D-glucopyranoside and methyl-α-D-mannopyranoside reduced ConA-DNA-complex formation, but they were not able to cause a complete inhibition of the binding (Fig. 05). These findings indicated that the sugar binding site somehow played a role in the interaction, but its occupancy by specific sugars did not completely inhibit ConA binding to DNA. Since the experiments were performed in buffer (Tris 10 mM, pH7.5) without metal ions, one could assume that a limited amount of ConA molecules were in the locked conformation and were able to bind saccharides (Brewer et al., 1974; Sherry et al., 1978; Stark and Sherry, 1979). But, earlier studies have demonstrated, that the equilibrium between the locked and unlocked states in metal-free ConA solutions, can be shifted by the addition of specific sugars. The addition of α-methyl-D-mannoside to metal-free ConA in solution, containing about 13% locked ConA under the given conditions, increases the amount of locked ConA to 60% (Brewer et al., 1983a). Hence, the observed partial inhibition of complex formation could be the result of an increase in the amount of locked ConA in solution. The weak inhibition of DNA binding by sugars could also be due to steric hindrance rather than to interference with the sugar-binding site (Becker et al., 1971). An incubation of ConA with trimannosides, which occupy a greater space in the ConA molecule, might give an answer for this question (Naismith and Field, 1996; Dam et al., 1998). A possible steric hindrance mechanism of inhibition would agree with the observation that, once ConA has bound to DNA, the subsequent addition of specific sugars, did not displace bound DNA from the ConA-DNA-complex (Fig. 05 B). In the ConA-tetramer, each monomer is not equally occupied by metal ions, and has not necessarily the same affinity for sugar (Brewer et al., 1974; Sherry et al., 1978; Stark and Sherry, 1979). Therefore, a ConA-tetramer that bound to DNA would still be able to bind to sugars through the remaining monomers of the tetrameric molecule. Evidence for this possible model of binding has been provided by the results of affinity chromatography and agglutination assays. ConA-DNA-complexes bind to a Sephadex column and can be eluted by glucose or mannose solutions, but not by galactose (Fig. 20, 21 and 22).

The partial inhibition of lectin-DNA-complex formation caused by α-methyl-D-glucopyranoside or α-methyl-D-mannopyranoside was also observed with two other lectins of the *Leguminosae* family (Fig. 34 B and C). The DNA-binding activity of the lectin from *Canavalia bonariensis* was not reduced by the presence of these sugars (Fig. 34 A). There was no proof that the sugar-binding site of the studied lectins was involved in DNA-binding. But the observation that the vast majority of the DNA-binding lectins was glucose/mannose-specific was quite intriguing (see Appendix, Table 01). The only DNA-binding lectin that was not glucose/mannose-specific was the PHA-E which recognized complex glycans. The lectins that did not bind to DNA (VVLB4, PNA and SBA) are galactose or galactosamine-specific. Whether a correlation between sugar specificity and DNA-binding ability existed, is a quite interesting question to be investigated.

5. 3. 2. The inhibitory effect of ATP

In order to investigate which component of the DNA-molecule would be recognized by ConA, the formation of ConA-DNA-complexes in the presence of nucleotide (ATP) or 2-D-deoxyribose was tested. Interestingly, ATP strongly inhibited complex formation (Fig. 06) while 2-D-deoxyribose did not (Fig. 07). ATP had a similar effect on the complex formation by leguminous lectins close related to ConA (Fig. 35). The ATP inhibition pattern of ConBn, ConBr and ConM (Fig. 35) was so similar to the pattern of ConA (Fig. 06), that even the range of inhibitory ATP-concentrations was the same. Hence, the bases and/or the phosphates in the DNA can be considered likely candidates to mediate the binding to lectin molecules. Many investigators have shown that a number of legume lectins (DBL, LBL, PHA-E, PHA-L, SBA and others) possess a binding site for adenine and adenine-derived plant hormones (Maliarik and Goldstein, 1988; Hamelryck et al., 1996; Loris et al., 1998; Bouckaert et al., 1999). Such an adenine binding site has not been described for ConA. It is doubtful that the adenine binding site found in the lectins mentioned is related to the ATP-inhibitory effect on lectin-DNA binding. The adenine binding sites are localized in cavities inside the tetramer structure or close to the dimer-dimer interfaces (Hamelryck et al., 1996). Moreover, the lectins SBA and PHA-L, which are reported to possess adenine-binding sites, are not able to form lectin-DNA-complexes. To evaluate the contribution of adenine in the ATP-inhibitory effect it is necessary to consider the structure of this nucleotide isolated and in comparison with the DNA structure. In ATP, the adenine-component exposes a number of groups (hydrogen donors or acceptors), which can be involved in a range of binding interactions (Bloomfield et al., 2000). In DNA, the base paaring of adenine with thymine reduces the availability of binding groups.

The overall tertiary structure of leguminous lectin subunits shares some structural similarities to a broad range of non-related proteins from a variety of organisms (Srinivasan et al., 1996; Loris et al., 2002). These structural similarities have often been used to search for new proteins in Genome Data Bases and to establish functional comparisons among various proteins. The observation that ATP inhibited ConA-DNA binding (Fig. 06) has encouraged a literature search for nucleotide-binding proteins that might share similarities to ConA. Some proteins that share structural homologies to ConA were worth analyzing. An example is a protein from *Methanobacterium thermoautotrophicum* (RmlC), which is an enzyme involved in the biosynthesis of the bacterial cell wall (Christendat et al., 2000). The enzyme RmlC is a dTDP-4-keto-6-deoxy-D-hexulose 3,5-epimerase which converts dTDP-4-keto-6-deoxy-D-glucose to dTDP-4-keto-L-rhamnose. The structural analysis of RmlC revealed significant structural homology to ConA. The high homology of RmlC to ConA comprises the jelly role structural motif of this enzyme with the overall core topology of ConA. The binding of RmlC to dTDP is stabilized by ionic interactions to the phosphate group and by hydrophobic and ionic interactions with the base. It will be interesting to analyze common features of the nucleotide binding-site of RmlC, which has been crystallized in a complex with dTDP, and the subunit structure of ConA. A superposition of the structures of ConA and RmlC might give an approximation of the position of the DNA-binding site of ConA (Christendat et al., 2000).

5. 3. 3. ConA-DNA-complex formation in the presence of 2-D-deoxyribose

The observation that the sugar 2-D-deoxyribose did not inhibit the formation of ConA-DNA-complexes did not necessarily imply that this sugar was not participating in lectin-DNA-binding. The conformation of free 2-D-deoxyribose in solution is distinct from its conformation in ATP or DNA (Altona, 1982). The conformation of the 2-D-deoxyribose in nucleotides depends on the torsion angles for rotation around each bond (Blomfield et al., 2000). In the DNA, three of the five carbons of the 2-D-deoxyribose are covalently linked to other groups. Carbon-1 of the 2-D-deoxyribose is linked to the base, carbon-3 to the phosphate (3'prime) and carbon-5 to the other phosphate (5'prime) (Altona, 1982). Because of the fixed position of the base in the helix and the continuity of the sugar-phosphate backbone in DNA, the rotation of the torsion angles is even more limited. Regarding the characteristics of the 2-D-deoxyribose as a component of nucleotides or DNA, the possibility that this sugar is involved in lectin-DNA interactions should not be underestimated.

5. 3. 4. The effect of anti-ConA-antibody on ConA-DNA-complexes

The incubation of ConA with anti-ConA-antibody led to a partial inhibition of ConA-DNA-complex formation (Fig. 08). The amount of antibody applied was probably not sufficient to cause complete inhibition. The relative low inhibitory effect of anti-ConA-antibodies could also be due to a dilution effect caused by free ConA. A large amount of ConA remained as free-ConA after ConA-DNA-complexes were formed (Fig. 24, 25 and 26B). In the antibody inhibition assay, ConA was first incubated with antibody, and the DNA was added subsequently. The amount of free-ConA that bound to the antibody might have interfered in the ConA-DNA binding equilibrium. Therefore, the presence of free-ConA during the inhibition reactions complicated the analysis of the obtained results. Certain antibody concentrations caused a "supershift" of the complexes which probably resulted from antibody-ConA-DNA complex formation.

5. 4 Properties of ConA-DNA-complexes related to the multivalency of sugar binding

The multivalency of sugar binding is one of the most interesting characteristics of leguminous lectins. The multimeric nature of these proteins allows multiple ligand binding to the same lectin molecule. The ability to bind to more than one ligand at the same time is the base of lectin-induced glycan precipitation (Agrawal and Goldstein, 1967b) and cell agglutination (Nonnan and Burger, 1973). Furthermore, a lectin can e.g. bind to the carbohydrate moieties of a glycoprotein, and this lectin/glycoprotein-complex can subsequently bind to a polysaccharide-matrix of a chromatographic column (Agrawal and Goldstein, 1967b). Based on these principles, experiments have been designed to investigate whether the lectin multivalency of sugar binding is preserved in ConA-DNA-complexes.

ConA-DNA-complexes were able to bind to a polysaccharide-based matrix like Sephadex and were specifically eluted by glucose or mannose (Fig. 20, 21 and 22). The DNA-shift observed in the eluted fractions attested to the stability of the ConA-DNA-complexes. The unbound fractions (peak I) and the retained fractions (peak II) contained ConA-DNA-complexes. Sephadex chromatography using ConA-DNA-complexes, which were prepared under a variety of ConA/DNA molecular ratios, gave always rise to DNA-containing peaks I and II. Therefore, it

was unlikely that unbound ConA-DNA-complexes were only due to an overload of the Sephadex columns. The presence of ConA-complexed DNA in peak I and II rather indicated that ConA-DNA-complexes were not homogenous in their ability to bind to Sephadex column. The observed heterogeneity of ConA-DNA-complexes was not due to different DNA-conformations present in ConA-DNA-complexes, because all DNA plasmid-forms, supercoiled, nicked and olygomeric were detected in both peaks. The most probable explanation for the heterogeneity of ConA-DNA-complexes is related to the degree of ConA metallization prior to ConA-DNA-complex formation.

The occupancy of each ConA subunit by metal ions (Ca^{2+} and Mn^{2+}) is decisive for the ability of lectin to bind to carbohydrate (Karlstam, 1973; Becker et al., 1975; Stark and Sherry, 1979). Partially metallized ConA binds with lower affinity and demetallized ConA (unlocked) does not bind to sugars (Stark and Sherry, 1979). Experiments that involve ConA-mediated sugar binding should be carried out in the presence of metal ions, to ensure the full metallization of ConA (Karlstam, 1973). But these experimental conditions could not be applied in the experiments described here, because fully metallized ConA did not form ConA-DNA-complexes (Fig. 14 and 16 A). Demetallized and poorly metallized ConA bound with higher affinity to DNA and the formed ConA-DNA-complexes were characterized (Fig. 15 and 16). But ConA-DNA-complexes, formed with demetallized ConA, were not expected to conserve the sugar binding ability of ConA. Native and commercially available ConA are normally in the metallized form, but they gradually lose their metal ions when solubilized and maintained in buffers devoid of metal ions (Uchida and Matsumoto, 1972; Karlstam, 1973; Willians et al., 1978). Therefore, under metal-free buffer conditions, native ConA gives rise to a mixture of ConA-molecules, which contains fully metallized, partially metallized and demetallized forms. The heterogeneity of such a ConA solution is further increased by the metal ions lost independently by each subunit (Sherry et al., 1978; Stark and Sherry, 1979). Since binding reactions were carried out in metal free buffer (10 mM Tris, pH7.5), it is likely that the formed ConA-DNA-complexes were heterogeneous with respect to their degree of metallization. ConA tetramers, composed of subunits with different degrees of metallization, might be able to bind to DNA and subsequently to Sephadex. These tetramers are probably responsible for the retention of ConA-DNA-complexes on the Sephadex-column (peak II). On the other hand, ConA tetramers, composed of demetallized or poorly metallized subunits, are probably responsible for the unbound ConA-DNA-complexes (peak I). ConA-DNA-complexes, formed with demetallized ConA, are not retained by Sephadex (Fig.23).

Agglutination assay is a widely used method to estimate lectin activity. In hemagglutination assays, erythrocytes are used. Because agglutination extent is proportional to the amount of lectin present, an approximate quantification of the lectin concentration is possible (Vlodavsky and Sachs, 1975; Doyle and Keller, 1984). Since lectin-induced agglutination requires the participation of more than one sugar-binding site in the same lectin molecule, agglutination extent somehow relates to the availability of multiple binding sites. The agglutination induced by a given lectin is inhibited by the presence of its specific sugars which occupy the binding sites necessary for the agglutination to occur (Agrawal and Goldstein, 1967a). Agglutination efficiency might also be affected by the interaction of the lectin with non-sugar ligands. A reduction in agglutination likely indicates the occupancy or steric block of some of the sugar binding sites by the ligand. In the case of lectin-DNA-complexes, the ligands are the DNA-molecules that are complexed with the lectin. To test the influence of DNA-binding on agglutination efficiency, ConA was incubated with different amounts of DNA followed by exposure to erythrocytes. Hemagglutination was detected in the presence of ConA-DNA-complexes (Fig. 18). The extent of agglutination was proportional to the molecular ratio ConA/DNA. The results indicated that part of the ConA-molecules exposed to the erythrocytes was no longer available to mediate agglutination. A possible explanation for these results was sought in the failure of ConA-DNA-complexes to mediate agglutination. The residual agglutination was possibly due to unbound ConA. This notion was supported by the finding that ConA-DNA-complexes (peak I) did not cause agglutination, whereas free-ConA (peak II) did (Fig. 25).

Cell agglutination can be seen as an effect of lectin binding at the macroscopic level. Considering the consequences of lectin binding at the level of a single cell, a number of intracellular reactions are reported. The mitogenic activity of some leguminous lectins e.g. is a consequence of lectin binding to cell membrane receptors (glycoproteins), followed by the activation of signal transduction pathways. The cell surface glycoprotein PZR e.g. was shown to be the major mediator of ConA-induced cell signaling (Zhao et al., 2002). Another consequence of cellular lectin binding is the internalization of the lectin-receptor complex by endocytosis. The surface and intracellular localization of ConA in human lymphocytes have been well characterized (Barat et al., 1973). It has been shown that ConA's uptake occurred by active pinocytosis. Inside the cell, ConA accumulate in cytoplasmatic vesicles that latter are fused with lysosomes. ConA-induced alteration of membrane cell dynamics and transport was reported to a number of cell types, including fibroblasts, hepatocytes and epithelial cells (Chowdhury, 1975; Huet and Bernadac, 1975; Roth and Binder; 1978). ConA also facilitates the internalization of proteins/glycoproteins that are bound/complexed to this lectin (Juliano et al., 1978, Storrie and

Maurey, 1981). Proteins that are internalized in complexes with ConA showed increased resistance against lysosomal degradation (Storrie and Maurey, 1981). The results obtained in Sephadex chromatography experiments showed the ability of ConA-DNA-complexes to bind to carbohydrates (peak II in Fig. 20, 21 and 22). In principle, ConA-DNA-complexes might also be able to bind to the glycoconjugates on the surface of cells and to be internalized. Based on this possibility, ConA-DNA-complexes (obtained from peak I of gel filtration in BioGel agarose) were exposed to mammalian cell lines in culture. The DNA-component of ConA-DNA-complexes was the pEGFP-C1 plasmid, which carried the gene for green fluorescence protein (GFP). Cells exposed to the ConA-DNA-complexes were analyzed for expression of GFP. As shown in Fig. 28, 29 and 30, some cells exposed to the complexes were green fluorescent, indicating the expression of the pEGFP-C1 plasmid. Despite the low efficiency of the observed gene transfer, GFP-expressing cells were only detected in the plates treated with ConA-DNA-complexes. Since specific staining experiments were not carried out, it could not be evaluated whether the internalized pEGFP-C1 plasmids were ConA-complexed.

5. 5 Lectin-DNA-complexes: biological significance and possible applications

The results presented in this investigation are the first reported for a nonspecific interaction of leguminous lectins with DNA. Since this interaction has not been previously reported, there is no information available about the possible biological significance of such interactions. Any discussion about a function(s) of this interaction *in vivo* is, therefore, highly speculative and has, so far, no experimental basis. Since the discovery of the first lectins, isolation and characterization of many lectins have been pursued all over the world. It is puzzling that the interaction lectin-DNA has not been detected before. The special *in vitro* conditions, required for the lectin-DNA binding reaction to occur, might explain why this interaction remained undiscovered over a long period of time. In the case of ConA, the lectin binds particularly well under conditions that are the opposite of those required for sugar binding. The interaction between lectin and DNA might exist in legume plants, but the standard lectin extraction procedures disrupt the binding in the process of lectin purification. In the legume seeds, lectins have been reported to interact with storage protein or enzymes mostly inside protein bodies. Those interactions are in some cases ionic and not related to the lectin sugar binding activity. It is unlikely that any leguminous lectin is associated with DNA inside the protein bodies, since DNA does normally not occur in this intracellular compartment.

The findings reported here raise important questions about the biological activities described for leguminous lectins. Although the analyses presented here have been carried out under *in vitro* conditions, they open the question, whether leguminous lectins, usually present in mammalian diets (Putztai et al., 1982; 1995; Dignass et al., 1996; Kordas et al., 2000), would be able to interact with DNA, protecting it against gastro-intestinal nucleases and possibly facilitating its survival and uptake by intestinal cells (Palka-Santini et al, 2003).

The ionic nature of the ConA-DNA-complexes indicates that the binding interaction between ConA and DNA is unlike to occur under physiological conditions (Fig. 17). Thus, in biological fluids like human blood plasma, at physiological NaCl concentrations, formation of ConA-DNA-complexes is unlikely to occur. The ionic strength inside cells is much more variable and has to be considered in dependency on cell type and tissue. Experiments using ConA-DNA-complexes for gene transfer in cell culture have shown that the ConA-DNA-complexes are stable enough to facilitate the expression of the reporter gene (GFP) after gene transfer into mammalian cells (Fig. 28, 29 and 30). Despite the observed low efficiency of this mode of gene transfer and low level expression of pEGFP-C1 plasmid using ConA-DNA-complexes, the results indicate that in principle the application of such complexes for gene transfer purposes is worth investigating.

Specific and sometimes also weak interactions of DNA with proteins encompass some of the most important reactions in biochemistry and molecular genetics. In past decades, special attention has been given to those protein-DNA-interactions, which would facilitate DNA transfer into cells (Wienhues et al., 1987). One of the intrinsic difficulties for DNA transfer into cells arises from the fact, that DNA is not easily bound and transported across cell membranes (Mahato et al., 1997). For this reason, any protein, which has the ability to interact with DNA and, at the same time, bind to receptors on the cell membrane, would be an ideal carrier to mediate the internalization of desirable DNA molecules into cells. The data presented here demonstrate that leguminous lectins, known to bind to the carbohydrate moieties of cell surface receptors, are also able to bind strongly to DNA. Lectin-DNA binding did not alter the DNA component and the DNA could be recovered intact from the lectin-DNA-complexes. Lectins have already been used in experiments on gene delivery, but manly as endogenous cellular receptors which facilitate the entry of glycosylated polycation-DNA-complexes or glycosylated liposomes (Erbacher et al., 1995; Shimizu et al., 1998; Fajac et al., 1999; Monsigny et al., 1999; Clarck et al., 2000; Yamazaki et al., 2000; Roche et al., 2003). The possibility exists, that DNA-lectin-complexes, generated experimentally, might provide another route by which foreign DNA could be transferred into eukaryotic, specifically into mammalian cells.

ABSTRACT

The interaction between leguminous lectins and DNA was investigated by using the 4.731 bp plasmid pEGFP-C1, which carried the gene of the green fluorescent protein. Supercoiled or nicked circular DNA molecules as well as subfragments of the whole plasmid were used as binding partners. In the binding assay, the lectin was incubated with DNA, and the formation of the lectin-DNA-complex was analyzed by electrophoresis in agarose gels. A concentration-dependent electrophoretic mobility shift of the DNA was observed with the lectins ConA, ConBn, ConBr, ConM, ConG, Dviol, Dvirg, LcA and PHA-E. No DNA binding activity could be detected for SBA, PHA-L, PNA and VVLB4 lectins. The binding of ConA to DNA was not sequence-specific since DNA of different sources and sizes bound this lectin. The kinetics of ConA-DNA-binding indicated that the complexes were formed very fast, probably in a matter of seconds. The size of the ConA-DNA-complexes was proportional to the ConA-concentration, but a saturation point of maximal binding could not be estimated. Upon protease digestion of the lectin in the DNA-lectin complex, the bulk of the DNA could be recovered intact. The interaction ConA-DNA was investigated under different pH and ionic conditions. A direct correlation between pH and complex formation was observed. Moderate ionic strength inhibited ConA-DNA complex formation, and an already formed complex was disrupted by the subsequent adjustment to high ionic concentrations. A pre-incubation of ConA with metal ions Ca^{2+}, Mn^{2+} or Mg^{2+} reduced dramatically the formation of ConA-DNA-complexes. EDTA influenced the formation of the complex with DNA and, for a range of concentrations, complex formation was enhanced. The pre-incubation of ConA with 2-D-deoxyribose, the sugar component of the DNA, had no effect on the extent of complex formation. ATP strongly inhibited complex formation, but was not able to disrupt preformed complexes. A previous exposition of ConA to α-methyl-D-glucopyranoside and/or to α-methyl-D-mannopyranoside reduced complex formation, while galactose did not. ConA-DNA-complex formation in the presence of anti-ConA-antibodies altered the electrophoretic migration of the complexes. ConA-DNA complexes were able to bind to a Sephadex column and could be eluted with glucose and/or mannose solutions. ConA submitted to dialysis against EDTA-containing buffers was able to bind to DNA but the formed complexes were not able to bind to the Sephadex column. The hemagglutination titer obtained after ConA-DNA-complex formation was reduced in comparison to the titer obtained by ConA alone. Upon ConA-DNA complex formation, DNA-bound ConA (peakI) could be separated from

free-ConA (peak II) by gel filtration on a BioGel agarose column. Whereas the DNA-bound ConA did not agglutinate erythrocytes, free-ConA conserved its ability to cause hemagglutination. CD-spectrum analyses of BioGel-purified ConA-DNA-complexes confirmed the presence and the relative abundance of DNA and ConA in the complex. Preliminary experiments to target ConA-DNA-complexes to mammalian cells in culture indicated successful transfer and expression of the pEGFP-C1 reporter plasmid in a limited number of cells. The biological importance and possible applications of lectin-DNA-complexes was discussed.

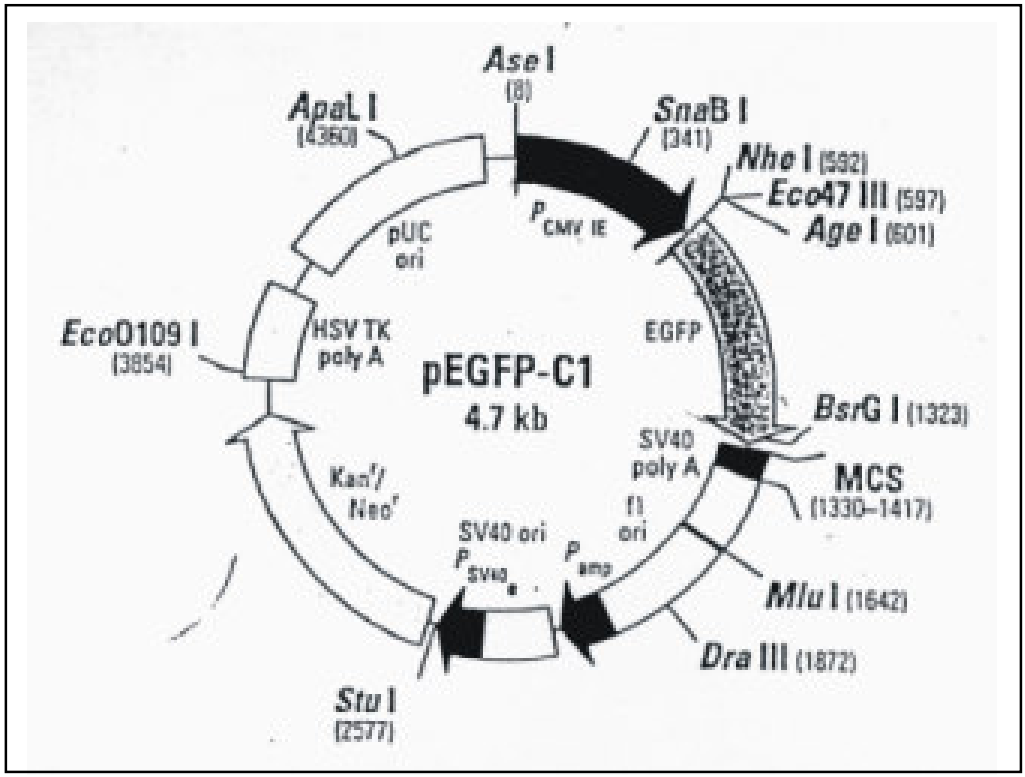

Figure 36: Schematic representation of the pEGFP-C1 plasmid. (CLONTECH Laboratories)

Table 01: Carbohydrate binding specificities of leguminous lectins[a]

Tribe	Species	Lectin abbrev.	Carbohydrate specificity[b]
Diocleae	*Canavalia ensiformis*	ConA	Man/Glc
	Canavalia brasiliensis	ConBr	Man/Glc
	Canavalia bonariensis	ConBn	Man/Glc
	Canavalia maritima	ConM	Man/Glc
	Canavalia grandiflora	ConG	Man/Glc
	Dioclea virgata	Dvirg	Man/Glc
	Dioclea violacea	Dviol	Man/Glc
Phaseoleae	*Phaseolus vulgaris*	PHA-E	Complex
	Phaseolus vulgaris	PHA-L	Complex
Stylosantheae	*Arachis hypogeae*	PNA[c]	Gal
Viceae	*Lens culinaris*	LcA	Man/Glc
	Vicia villosa B4	VVLB4	GalNac
Glycineae	*Glycine max*	SBA[d]	Gal / GalNac

[a]Legume lectins used in this investigation.

[b]Man/Glc (mannose/glucose); Gal (galactose); GalNac (N-acetylgalactosamine).

[c]Peanut agglutinin.

[d]Soybean agglutinin.

Table 02: Association constants of the interaction of some lectins with carbohydrates.

Species	K_{Ass} M^{-1}	Carbohydrate
Canavalia ensiformis (Jack bean)	0.8×10^4	α-methylmannopyranoside
Glycine max (Soybean)	1.4×10^4	galactose (α)
Arachis hypogeae (Peanut)	2.0×10^3	lactose
Pisum sativum (Pea)	1.3×10^3	mannose (α)
Lens culinaris (Lentil)	5.6×10^2	α-methylmannopyranoside

(from Rüdiger, 1998)

Table 03: Stoichiometry of ConA and DNA in the complexes*. Binding reactions to investigate ConA-DNA-complex formation. (Fig. 01, 09A, 12, 15A).

ConA (ng/µl)	DNA (ng/µl)	Molar ratio
9 000	6	170 586
6 750	"	127 939
5 063	"	95 955
3 797	"	71 966
2 848	"	53 975
2 135	"	40 481
1 602	"	30 361
1 201	"	22 770
901	"	17 078
676	"	12 808
506	"	9 606
380	"	7 205
285	"	5 404
214	"	4 053
160	"	3 039

*Molar ratios have been calculated on the basis of molecular weights of 3.07×10^6 for pEGFP-DNA and 27,000 for the monomeric subunit of ConA.

Table 04: Stoichiometry of ConA and DNA in the complexes*. Binding reactions to investigate complex inhibition, kinetics of binding and the influence of ions on complex formation. (Fig. 05, 06, 07, 08, 10, 14, 15 B and 17).

ConA (ng/µl)	DNA (ng/µl)	Molar ratio
1 200	6	22 745

*Molar ratio has been calculated on the basis of molecular weights of 3.07 x 10^6 for pEGFP-DNA and 27,000 for the monomeric subunit of ConA.

Table 05: Stoichiometry of ConA and DNA in the complexes*. The formation of ConA-DNA-complexes using pEGFP-C1-subfragments or DNA of other sources as binding partners. (Fig. 09 B and C).

ConA (ng/µl)	DNA (ng/µl)	Molar ratio
9 000	16.7	61 288
6 750	"	45 966
5 063	"	34 475
3 797	"	25 855
2 848	"	19 392
2 135	"	14 544
1 602	"	10 908
1 201	"	8 181
901	"	6 135
676	"	4 602
506	"	3 451
380	"	2 589
285	"	1 941
214	"	1 456
160	"	1 029

*Molar ratios have been calculated on the basis of molecular weights of 3.07 x 10^6 for pEGFP-DNA and 27,000 for the monomeric subunit of ConA.

Table 06: Stoichiometry of ConA and DNA in the complexes*. Binding reaction to test the influence of a range of DNA-concentrations on ConA-DNA-complex formation. (Fig. 11).

sample	ConA (ng/µl)	DNA (ng/µl)	Molar ratio
01	9 000	200.00	5 117.58
02	"	100.00	10 235.16
03	"	50.00	20 470.32
04	"	25.00	40 940.64
05	"	12.50	81 881.28
06	"	6.25	163 762.56
07	"	3.13	327 525.12
08	"	1.56	655 050.24
09	"	0.78	1 310 100.48
10	"	0.39	2 620 200.96

*Molar ratios have been calculated on the basis of molecular weights of 3.07 x 10^6 for pEGFP-DNA and 27,000 for the monomeric subunit of ConA.

Table 07: Stoichiometry of ConA and DNA in the complexes*. Binding reaction for the experiments to test the recovery of DNA out of ConA-DNA-complexes. (Fig. 13)

ConA (ng/µl)	DNA (ng/µl)	Molar ratio
9 000	6	170 586
1 200	"	22 745
160	"	3 033

*Molar ratios have been calculated on the basis of molecular weights of 3.07 x 10^6 for pEGFP-DNA and 27,000 for the monomeric subunit of ConA.

Table 08: Stoichiometry of ConA and DNA in the complexes*. Binding reactions to analyze ConA-DNA-complex formation by using TE-dialyzed ConA. (Fig. 16 A).

ConA (ng/µl)	DNA (ng/µl)	Molar ratio
3 000.0	6	56 862
1 500.0	"	28 431
750.0	"	14 216
375.0	"	7 108
187.5	"	3 554
97.8	"	1 777
46.9	"	888
23.4	"	444

*Molar ratios have been calculated on the basis of molecular weights of 3.07 x 10^6 for pEGFP-DNA and 27,000 for the monomeric subunit of ConA.

Table 09: Stoichiometry of ConA and DNA in the complexes*. Binding reactions to analyze ConA-DNA-complex formation by using TE-dialyzed ConA. (Fig. 16 B and C).

ConA (ng/µl)	DNA (ng/µl)	Molar ratio
9 000.0	6	170 586
4 500.0	"	85 293
2 250.0	"	42 647
1 125.0	"	21 323
562.5	"	10 661
281.3	"	5 331
140.6	"	2 665
70.3	"	1 332
35.2	"	666
17.6	"	333
8.8	"	167
4.4	"	83
2.2	"	42

*Molar ratios have been calculated on the basis of molecular weights of 3.07 x 10^6 for pEGFP-DNA and 27,000 for the monomeric subunit of ConA.

Table 10: Stoichiometry of ConA and DNA in the complexes*. ConA-DNA-complexes prepared for chromatographic experiments. (Fig. 20, 21, 22, 23, 24, 25, 26 and 27).

Fig.	ConA (ng/µl)	DNA (ng/µl)	Molar ratio
20	2 670	8.6	35 141
21	1 200	12.0	11 372
22	3 000	15.0	22 745
23	2 000	10.0	22 745
24	10 000	100.0	11 372
25	5 000	100.0	5 686
26	10 000	100.0	11 372
27	6 666	66.6	11 372

*Molar ratios have been calculated on the basis of molecular weights of 3.07×10^6 for pEGFP-DNA and 27,000 for the monomeric subunit of ConA.

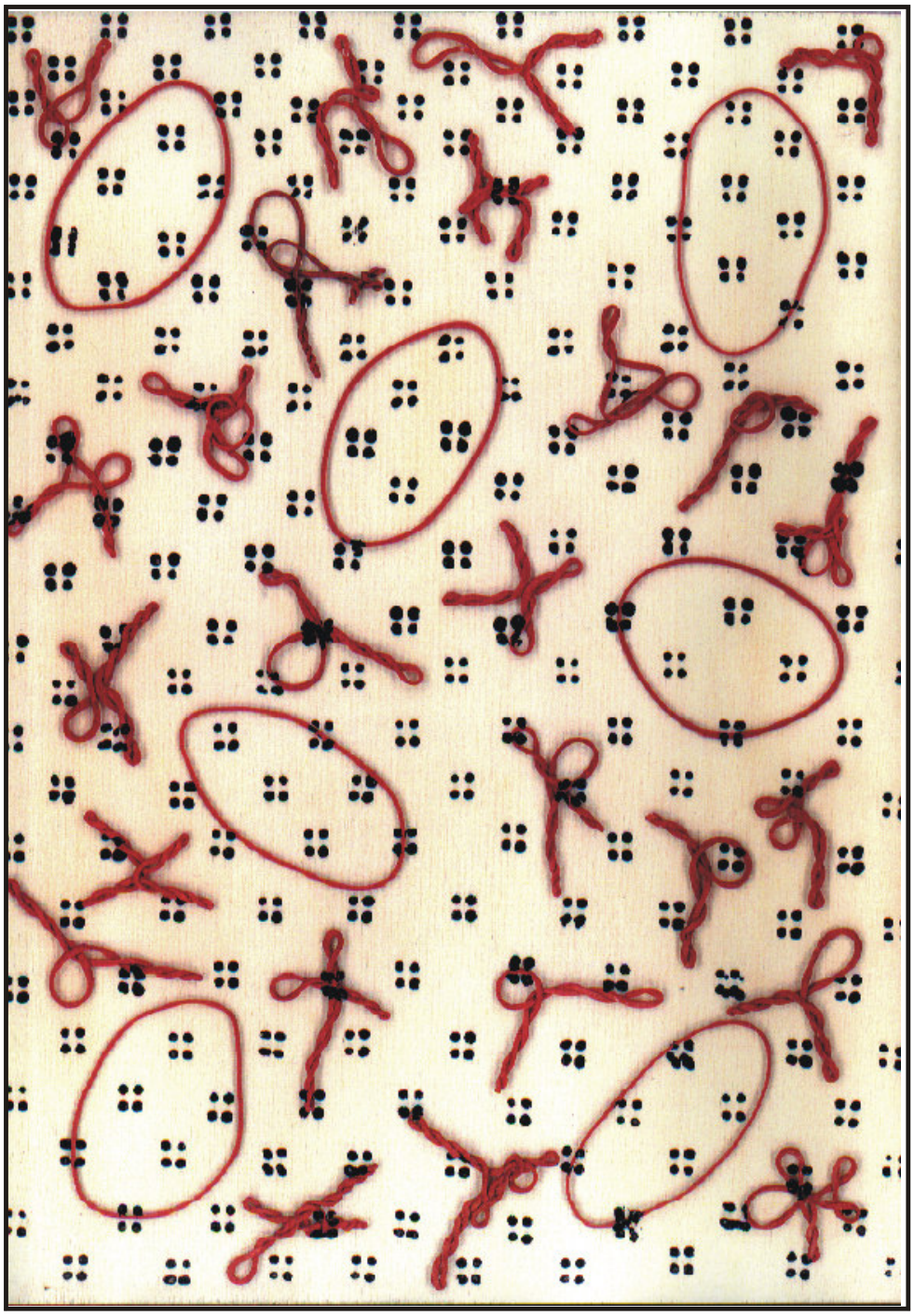

Figure 37: Artistic representation of lectin binding to plasmid DNA-molecules.

(Mixed technique with acryl and plastic rings on wood plate, 30 x 21 cm)

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ZUSAMMENFASSUNG

Die Interaktion zwischen *Leguminosae* Lektinen und DNA wurde mit dem 4.731 Basenpaar langen pEGFP-C1 Plasmid, welche das Gen für das grün fluoreszierende Protein kodiert, untersucht. Supercoiled oder genickte zirkuläre DNA Moleküle, sowie Fragmente des gesamten Plasmids wurden als Bindungspartnern verwendet. Während der Bindungsreaktion wurden die Lektine mit der DNA inkubiert und die entstandenen Lektin-DNA-Komplexe wurden durch Agarose Gel Elektrophorese analysiert. Eine konzentrationsabhängige elektrophoretische Retardierung der DNA wurde bei den Lektinen ConA, ConBn, ConBr, ConM, ConG, Dviol, Dvirg, LcA und PHA-E beobachtet. Es konnte keine DNA bindende Aktivität bei den Lektinen SBA, PHA-L, PNA und VVLB4 beobachtet werden. Die Bindung von ConA an DNA war nicht sequenzspezifisch, weil DNA unterschiedlicher Länge und Herkunft von diesem Lektin gebunden werden konnte. Die Kinetik der ConA-DNA Bindungsreaktion weist darauf hin, dass die Reaktion sehr schnell erfolgte, möglicherweise innerhalb von Sekunden. Die Grösse der ConA-DNA-Komplexen war proportional zu der Konzentration von ConA, aber eine Sättigungskonzentration der maximalen Bindung konnte nicht ermittelt werden. Die DNA konnte durch proteolytischen Behandlung aus den ConA-DNA–Komplexe in intakter Form isoliert werden. Die Interaktion ConA-DNA wurde unter unterschiedlichen pH Werten und ionischen Bedingungen untersucht. Eine direkte Korrelation zwischen pH-Werten und Komplexbildung konnte beobachtet werden. ConA-DNA-Komplex Bildung wurde durch milde ionische Konzentrationen inhibiert, und gebildete Komplexe konnten durch eine Erhöhung der Ionenkonzentration zerstört werden. Eine vorherige Inkubation von ConA mit den Metallionen Ca^{2+}, Mn^{2+} oder Mg^{2+} führte zu einer Verringerung der ConA-DNA-Komplexbildung. EDTA hat die Bildung von ConA-DNA-Komplexen beeinflusst und für eine Reihe von EDTA-Konzentrationen war die Komplexbildung sogar verstärkt. Eine Vorinkubierung von ConA mit 2-D-Deoxyribose, die Zuckerkomponente der DNA, hatte keinen Einfluss auf die Komplexbildung. ATP hat die Bildung von ConA-DNA-Komplexen stark inhibiert, aber bereits gebildete Komplexe konnte nicht durch Zugabe von ATP zerstört werden. Eine vorherige Inkubierung von ConA mit α-methyl-D-Glucopyranosiden oder α-methyl-D-Mannopyranosiden hat die Komplexbildung stark reduziert, Galaktose jedoch nicht. ConA-DNA-Komplexbildung in Anwesenheit von Anti-ConA-Antikörper führte zu eine Veränderung der elektrophoretischen Mobilität des Komplexes. ConA-DNA-Komplexe waren in der Lage an eine Sephadexsäule zu binden und wurden durch Zugabe von Glucose oder Mannose Lösungen eluiert. ConA-

Moleküle, die gegen eine EDTA-haltige Pufferlösung dialysiert wurden, konnte an DNA binden, aber die entstandene Komplexe konnten nicht an die Sephadexsäule binden. Der Hemagglutinationstiter nach ConA-DNA-Komplexbildung war im Vergleich zu dem durch ConA alleine verursacht Titer reduziert. Eine Trennung von DNA-gebundenem ConA (Peak I) und freiem ConA (Peak II) konnte durch Chromatographie in BioGel Agarose Säule erreicht werden. Während das freie ConA Erythrocyten agglutinieren konnte, konnte das DNA-gebundene ConA keine Agglutination verursachen. CD-Spektrum Analyse von BioGel gereinigten ConA-DNA-Komplexen bestätigten die Anwesenheit und die relative Mengen von DNA und ConA in den Komplexen. Anfängliche Experimente zur Übertragung der ConA-DNA-Komplexe in kultivierte Säugerzellen haben gezeigt, dass das pEGFP-C1 Reporterplasmid in eine begrenzten Anzahl von Zellen erfolgreich aufgenommen und exprimiert wurde. Die biologische Relevanz und die mögliche Anwendungen von Lektin-DNA-Komplexen wurden diskutiert.

ERKLÄRUNG

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stelle der Arbeit – einschließlich Tabellen, Karten und Abbildungen - , die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie noch nicht veröffentlich worden ist sowie dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Herrn Professor Dr. Walter Doerfler betreut worden.

Ich versichere, dass ich alle Angabe nach bestem Wissen und Gewissen gemacht habe und verpflichte mich, jedmögliche, die obigen Angaben betreffend Veränderungen, dem Dekanat unverzüglich mitzuteilen.

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