CHAPTER 15

IMMUNOREACTIVE PROTEINS: THEIR PRODUCTION AND IMPORTANCE IN HAEMOPROTOZOAN DISEASES

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INTRODUCTION

Most epidemiological studies on haemoparasitic infections in veterinary medicine are concerned with the prevalence. Serological methods have been used to diagnose haemoparasitic infections, which have been mainly diagnosed with traditional methods for many years, since the 1990s. In recent years, the prevalence of haemoparasites can be precisely determined by DNA-based methods. These methods are valuable research tools, but costly and time-consuming for large-scale epidemiological studies. Therefore, it is necessary to develop sensitive and cost-effective diagnostic methods, even applicable in the field, to implement effective control strategies in protozoan infections. Since parasite antigens prepared from infected tissues contain some host proteins, false-positive reactions are encountered in serological tests using these antigens. Immunoreactive proteins obtained using recombinant DNA technology are more purified and do not carry host factors, so the speed, specificity, and sensitivity of tests using these proteins are very high. These proteins can be easily used as antigens in a quantitative test such as ELISA or an immunochromatographic test that can be applied even in the field. Immunoreactive proteins are also significant as vaccine candidates in controlling infections (Kumar et al. 2002; Huang et al. 2006; Terkawi et al. 2007; Ooka et al. 2012).

Serological methods are widely used in epidemiology. However, no commercial product is used for the serodiagnosis of all parasitic diseases. ELISA and IFAT are the most widely used serological methods in the diagnosis of protozoan diseases. The antigens used in the IFAT are prepared with parasites grown in culture or obtained from an infected host. The disadvantages of the IFAT include the need for an expert and a fluorescent microscope during the evaluation phase, the observation of cross-reactions between some species, and the long evaluation phase. Therefore, it is essential to develop parasite-specific antigens and to use them in diagnostic methods suitable for large-scale epidemiological studies. ELISA finds more widespread use in epidemiological studies due to its automation, no need for an expert, objective and quantitative evaluation of the results, the possibility of examining many samples together, and its higher specificity compared to the IFA test. Various types of ELISA are used to diagnose protozoan species such as *Babesia ovis, Toxoplasma gondii, Neospora caninum, Cryptosporidium parvum.* In the past, crude antigens prepared from infected animal blood were used in ELISA for the indirect diagnosis of haemoprotozoan infections. Accordingly, high cross-reactions with serum proteins were observed during the test. This situation has recently disappeared with the use of recombinant immunoreactive proteins as antigens in ELISA tests, and the specificity and reliability of ELISA have been increased (Duzgun et al. 1991; Sevinc et al. 2015a; 2015b; Ceylan and Sevinc 2020).

Studies on *Plasmodium* and some *Babesia* species have revealed the host immune system reaction against the merozoite proteins of these protozoa. Based on this, great importance has been given to studies to determine the immunoreactive proteins of parasites. Immunoreactive proteins are of great importance both as antigens in indirect diagnosis and as vaccine candidates that can be used in protection against the disease since they are the parts of the parasite stimulating the host immune system. Recombinant proteins are synthesized in vitro with the help of recombinant DNA technology. Parasite's immunoreactive proteins are being replicated by inserting genes into suitable vectors and transferring these vectors to a suitable host (Brown et al. 2006; Terkawi et al. 2007; Temizkan and Gozukirmizi 2018).

The development of new and practicable diagnostic methods and protective vaccines against diseases is one of the priority areas in veterinary research. This book chapter discusses recent developments in recombinant proteins and their role in diagnosing protozoan diseases and vaccine development. The first and most important step in this area is the selection of proteins or antigens to be used during the serological examination or in the vaccine development. For this purpose, various proteomic, genomic, and immunological screening protocols are used. Sometimes this selection is more functional or requires more biological data. Once the candidate gene encoding a particular protein is identified, several key steps are initiated, including cloning, production, purification, validation, and assay design. The selected protein can be produced in a heterologous expression system or host cells grown under certain laboratory conditions. Among the alternatives, the most frequently used heterologous expression system is *Escherichia coli* (Brondyk et al. 2009; Sözen et al. 2010).

The usage of immunoreactive proteins in different fields

In parallel with the advances in molecular biology, additional diagnosis, treatment, and prevention methods can be developed in the field of health by various changes which have

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recently been made on micromolecules such as DNA, RNA, protein, and antibodies. Using recombinant DNA technology, molecules of a pathogenic microorganism can be synthesized at the desired time and amount. These can be used for both diagnostic methods and vaccine development. Recombinant antigens in diagnosing diseases have increased the speed, specificity, and sensitivity of diagnostic tests. There are many recombinant proteins used in veterinary medicine to diagnose diseases. Recombinant antigens determined as a result of immunological screening of cDNA libraries are utilized in most of the methods used in the diagnosis of diseases such as malaria, trypanosomiasis, babesiosis, and leishmaniosis (Lodish et al. 2004; Fernandez-Robledo and Vasta 2010).

Proteins are encoded by genes to carry out the life activities of living creatures. In the case of disease, the protein density of the organism changes, depending on the biological stages of the disease-causing agent in the host body. In such cases, it may be possible to solve the problems related to diseases by analyzing the structural properties, functions, and interactions of the synthesized proteins with other molecules. While parasites continue to develop after entering the host body, many immunogenic proteins are released by the parasite into the host body. Accordingly, the host immune system tries to inhibit the development of the parasite by activating defense factors against these proteins (Lodish et al. 2004; Konuk 2004; Brown 2009; Sözen et al. 2010).

The vast majority of vaccines used to protect against various organisms are live vaccines obtained by attenuation of pathogenic microorganisms or inactivated vaccines. Due to the risk of active infection in attenuated live vaccines or live vaccines with incomplete inactivation, immunoreactive proteins have been used as immune system stimulating factors in vaccination studies in recent years. When a protein vaccine is administered to an individual, the immune mechanism of the host becomes active against the epitopes in the protein's structure that makes the protein antigenic or immunogenic. The development of the infectious agent in the host is prevented by the antibodies formed against the protein antigens and the developing cellular immune reactions in the body of the vaccinated individual (Lodish et al. 2004; Konuk 2004; Brown 2009; Sözen et al. 2010).

Immunoreactive proteins of any pathogen are valuable products of medicine and veterinary health. They are used both as a specific antigen in the serological diagnosis and as immunogens activating the host's immune system. Proteins are expressed in low concentrations and are therefore difficult to isolate and purify in large quantities by standard biochemical techniques. To produce these proteins in sufficient quantities and carry out basic research on their structure and function, systems that can produce them in large quantities at a reasonable cost are required. Recombinant DNA techniques, which transform *E. coli* cells into factories synthesizing such proteins, are now utilized to produce human proteins used for therapeutic purposes, such as blood coagulation factor, granulocyte colony-stimulating factor, insulin and growth hormone. In veterinary medicine, they are primarily used to diagnose diseases and develop vaccines (Jenkins 2001; Brown et al. 2006; Ferrer-Miralles et al. 2009).

Identification of immunoreactive proteins of parasites

Producing immunoreactive proteins in sufficient quantity and purity is possible by cloning the genes encoding these proteins using recombinant DNA technology and synthesizing them in a heterologous expression system. The most important resources used to identify genes encoding immunoreactive proteins are DNA libraries (Temizkan and Gozukirmizi 2018).

DNA libraries

A researcher who uses recombinant DNA technology to clone a unique gene or DNA fragment must have sufficient knowledge of the gene of interest to achieve the goal successfully. Therefore, DNA libraries are needed. Firstly, DNA is isolated from the studied microorganism and subjected to the cutting process with restriction enzymes. DNA fragments obtained as a result of cutting are cloned in various vectors (λ cloning vector, BAC, YAC, etc.) to reveal many colonies carrying certain genome regions. All clones contain at least one copy of each gene in the genome from a DNA library. DNA libraries can be screened to detect a specific gene or clone containing DNA fragments. DNA libraries are also used to isolate some specific genes and genome organization. DNA libraries are separated into two groups, as genomic DNA (gDNA) library and complementary DNA (cDNA) library, according to the type of DNA prepared in these libraries. Genomic DNA libraries are assumed to represent the entire DNA sequences in an organism, whereas cDNA libraries contain only the transcribed parts of the genome (Sözen et al. 2010).

A specific gene can be removed from the libraries and can be studied. These libraries can be reproduced in need, used for many different purposes, and preserved for years. Since only the expressed genes in an organism are transcribed into mRNA, if the material cloned in the preparation of the DNA library is cDNA obtained from mRNA, the clones formed only cover a group of the total number of genes in the cell. The mRNA cloning method is more beneficial than gDNA if the desired gene is expressed in a particular cell type at high levels. mRNA cannot bind to the cloning vector. Therefore, after it has been converted into a double-stranded cDNA molecule, it can be ligated into a vector and cloned. The resulting cDNA clones represent mRNA. If the mRNA is prepared from a single-celled protozoan, the genes encoding all the proteins synthesized by the protozoan during development are largely contained in the cDNA library. It is a much more laborious process to detect the equivalent of a gene of cDNA library in the gDNA library (Brown 2009; Sözen et al. 2010).

Genomic DNA libraries

A gDNA library is a collection of clones thought to contain one copy of all DNA sequences in the genome. These libraries can be built in a variety of ways. At first, gDNA is cut with various restriction enzymes, and the resulting fragments are cloned. However, this method has several disadvantages. For example, the gene to be studied may have recognition sites for the restriction enzyme, and as a result, the gene may be undesirably fragmented and cloned separately. Another disadvantage is that the fragments obtained by cutting with the enzyme are small. This means that when large eukaryotic genomes are considered, the library to be created will contain many clones. This increases the workload of scanning the library for a specific gene and is not economical. To overcome such drawbacks, there is need to obtain longer DNA fragments to create a library with few clones, in which genes are placed in a

single clone as much as possible. It is possible to achieve this by partially cutting the gDNA with various enzymes (Sau3A, Mob1) with a 4-base pair recognition region and reducing the amount of enzyme placed in the medium and incubation time during this process. After cutting, DNA fragments representing the whole genome and having the desired size are collected by agarose gel electrophoresis and cloned directly. Various λ displacement vectors (EMBL4), cosmid vectors (pJ88, c2RB), artificial yeast chromosomes (YAC) and artificial bacterial chromosomes (BAC) vectors are used for cloning. Generally, bacteriophage and cosmid vectors are used to create gDNA libraries. After the DNA fragments are transferred into the selected vector, they are transferred to *E. coli* and amplified in a selective medium. It is assumed that each resulting colony contains a different DNA fragment clone. Then, by using various methods, studies can be carried out to reach the targeted gene from these gDNA libraries (Sözen et al. 2010; Allison 2014; Temizkan and Gozukirmizi 2018).

cDNA libraries

cDNA libraries are constructed using the parasite's mRNA molecules. These libraries are the most critical resource for detecting genes encoding immunoreactive proteins. When cDNA libraries are subjected to immunological screening, it can be determined that many genes in the library encode immunoreactive proteins. Once a specific gene is obtained, its molecular, genetic and immunological properties can be examined, and solutions can be developed for diseases (Brown et al. 2006). Since the RNase enzyme rapidly degrades single-stranded mRNAs, mRNA isolation in vitro is tough. Therefore, single-stranded mRNA molecules are converted to their DNA counterparts, cDNAs. cDNA libraries can be created by cloning cDNAs synthesized from mRNA. cDNA libraries allow us to have information about the active genes at the time of mRNA isolation from the studied cell type (Sözen et al. 2010; Temizkan and Gozukirmizi 2018).

To prepare a cDNA library, total RNA is isolated from tissues or cells of interest, followed by mRNA isolation. Various essential elements such as reverse transcriptase enzyme and oligo-dT primers are required to convert the isolated mRNA to cDNA. The restriction sites on the cDNA are methylated to protect them from cleavage by the restriction enzyme. Then the recognition site sequence of a restriction enzyme, which can form sticky ends, is added to both ends by the DNA ligase enzyme. Then, the ends of this cDNA double strand are cut with a restriction enzyme to obtain sticky ends. Meanwhile, the region of the cloning vector bacteriophage λ to which foreign DNA is inserted, is also cut with the same enzyme and the same sticky ends are obtained. Complementary λ-phage and cDNA assembly with sticky ends are mixed under appropriate conditions and covalently coupled with DNA ligase. Each of the resulting recombinant DNA molecules contains a piece of cDNA located between the two arms of the DNA of the λ vector. The virions containing the recombinant DNA are then packaged in vitro. After packaging, the recombinant λ-phages are transferred onto petri dishes coated with *E. coli* cells. Phages adhere to the membrane of tail *E. coli* cells, transfer the DNA molecule in the head region into the cell, and many new phages are formed inside the cell. The newly formed phages are released by dividing the cell and continue to multiply in the same way by entering other cells. Meanwhile, numerous individual plaques of transparent appearance are formed due to the disintegration of the cells. Since each plaque is derived from a single recombinant phage, all new phages formed in a plaque are genetically identical and form a clone carrying a cDNA derived from a single mRNA. The plaques formed are called a λ-cDNA library (Mullinax and Sorge 2003; Lodish et al. 2004; Brown 2009; Temizkan and Gozukirmizi 2018). The steps of constructing a cDNA library are shown in Figure 1.

Immunoscreening

After the cDNA library is created, it can be immunologically screened to identify various antigenic proteins. Serological screening of libraries from microbial pathogens plays an essential role in the development of new drugs and recombinant vaccines. Identification of a portion of the pathogen proteome recognized by the host immune system is essential in identifying epitopes of the pathogen that trigger protective immunity. Systematic mapping of complex parasite immunome is faster and more efficient than the methods initially developed for serological screening of expression libraries. Genomic and cDNA libraries of various organisms contain thousands of clones. Two general approaches are used to screen libraries to detect clones carrying a DNA region or gene region of interest. The first of these is the detection with the help of oligonucleotide probes that can bind to the clone; the second is the detection based on the expression of the encoded protein (Ardeshir et al. 1985; Sözen et al. 2010).

In screening a λ cDNA library, a nitrocellulose membrane is firstly placed on the petri dish containing many λ clones, and the clones then adhere to the membrane. The membrane is then tested using a radioactive-labeled probe specific for recombinant DNA containing the DNA fragment of interest. Once a cDNA clone encoding a specific protein is found, the full-length cDNA can be labeled with radioactive substances and used as probes to locate clones containing fragments of the gene of interest from the genomic library. The appearance of a spot on the autoradiogram indicates a recombinant clone λ containing the DNA complementary to the probe. The position of the spot in the autoradiogram is the mirror symmetry of the particular clone in the petri dish. Well-separated plaques can be formed by recoating the detected phage particles. Finally, pure isolates are obtained by repeating the hybridization method (Lodish et al. 2004).

Nowadays, various immunodominant or immunoreactive proteins belonging to protozoa can be obtained by scanning cDNA libraries prepared from many parasitic protozoa causing diseases in humans and animals. These proteins are then used in diagnostic studies and vaccine studies (Bose et al. 1990; Kappmeyer et al. 1999; Ikadai et al. 2000; Fukumoto et al. 2001; Boonchit et al. 2002; Goff et al. 2003; 2006; 2008).

Screening of cDNA library using specific antibodies

Recombinant protein production is complicated in practice. To produce large quantities of a protein, the cDNA clone encoding this protein must first be removed from the library. One of the procedures that enable the detection of the desired clone from the cDNA library is the hybridization-probing technique that directly identifies the recombinant DNA molecule. The main alternative to this technique, which has limited use, is immunological screening. The proteins encoded by the cloned gene are detected in the immunological screening method. Specific antibodies are

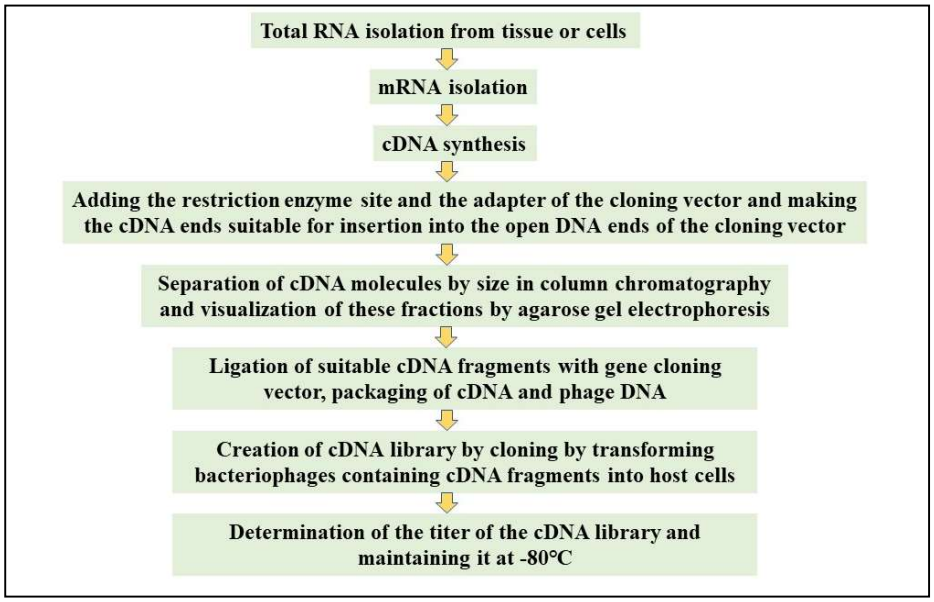

Figure 1. The steps of constructing a cDNA library.

needed to detect protein in recombinant colonies. After transferring the recombinant colonies to the polyvinyl or nitrocellulose membrane, they are treated first with the primary antibody solution and the enzyme-labeled secondary antibody. Positive colonies are then highlighted by using a substrate to express the enzyme. After the positive colonies in the medium are removed from the medium and cloned again in a suitable host, the relevant gene is extracted, the sequence information is determined and then it is possible to produce it in a bacterial expression system at any time and in desired quantities by placing it in an expression vector to produce a large amount of immunoreactive protein (Mullinax and Sorge 2003; Lodish et al. 2004; Brown 2009; Sözen et al. 2010; Bayraç et al. 2012; Temizkan and Gozukirmizi 2018).

Synthesis of proteins from genes encoding immunoreactive proteins

To produce recombinant proteins in large quantities, expression vectors must be constructed to synthesize the encoded protein when transferred into *E. coli* cells. Expression vectors, unlike cloning vectors, contain a promoter and a terminator region that can initiate protein synthesis. Transcription of the target gene begins at the promoter region and ends at the terminator sequence. The most abundant promoters in expression vectors are the Lac, Tac and T7 promoters. Most expression vectors have multiple cloning sites containing the ribosome binding sequence that provides efficient translation initiation and restriction enzyme cleavage sites. For example, a plasmid expression vector producing the β-galactosidase protein has a fragment of the *E. coli* chromosome containing the lac-promoter and the lac-z gene inserted in its DNA. When lactose or its analog isopropyl thiogalactoside (IPTG) is present in the culture medium, the RNA polymerase enzyme transcribes the lac-z gene starting from the promoter region, and lac-z-mRNAs are produced. The β-galactosidase protein is produced from these mRNAs. Protein production continues as *E. coli* cells proliferate in a culture medium containing IPTG. Thus, some eukaryotic proteins can be produced in *E. coli* cells using plasmid vectors containing promoter regions (Brondyk et al. 2009; Zerbs et al. 2009; Temizkan and Gozukirmizi 2018).

Product purity and high yield are critical in recombinant protein production. Therefore, eukaryotic proteins produced in *E. coli* expression systems are often synthesized as a fusion protein to facilitate differentiation from endogenous *E. coli* proteins. Most expression vectors produced in biotechnology carry expression tags such as glutathione S transferase (GST), maltose-binding protein, and histidine. Expression tags are required for the purification of the protein after translation. GST is one of the most commonly used expression tags in recombinant protein production. GST-labeled fusion proteins are expressed at high levels in the *E. coli* expression system. Recombinant proteins can be easily purified by glutathione affinity chromatography at the end of production (Hunt 2005; Malhotra 2009; Graslund et al. 2008).

The pGEX series plasmid vectors produced by different companies have been successfully used to produce proteins encoded by specific genes detected by immunological screening from cDNA libraries in *E. coli* expression systems. pGEX expression vectors carrying ampicillin resistance gene, tac promoter, lac suppressor gene region, and GST tag are among the most widely used plasmids for expressing proteins. When IPTG is added to the culture medium, the tac promoter region in these vectors is induced, and protein expression then begins (Ikadai et al. 1999; Fukumoto et al. 2001; Liu et al. 2010; Ooka et al. 2011; Luo et al. 2011; Cao et al. 2013).

Cloning vectors

In order to molecularly investigate the structure and functions of a gene, that gene must be purified in large quantities. The most important part of cloning a specific DNA is combining it with a vector DNA molecule. Vectors are intermediary molecules commonly used for gene cloning. The vector should have the ability to make copies in a suitable host cell. Recombinant DNA molecule consisting of vector DNA and a foreign DNA fragment attached to it, is transferred into a host

cell. This recombinant DNA replicates in the host cell, producing multiple identical copies called clones. As host cells proliferate and increase in number, recombinant DNA is also passed on to newly formed offspring, creating a population of identical cells carrying the cloned sequences. Then, the cloned DNA fragment can be retrieved from the host cell, purified, and used for various purposes such as gene expression, immunological screenings and sequence analysis (Sözen et al. 2010; Allison 2014).

DNA fragments obtained after cutting with restriction enzymes, cannot enter the bacterial cell directly under normal conditions. Therefore, they must be inserted into a vector DNA that can replicate in bacteria. The vectors used in cloning must have some properties. The vector should have small molecule structure. It should be easily isolated from bacteria and carry a suitable replication center for the host. Many enzymes must have only one recognition site in vector DNA. When this region is cut with an enzyme, it is used to insert the gene fragment cut with the same enzyme. Unlike these, vector DNA must contain a selective marker gene to distinguish between host cells with and without the vector, such as a gene responsible for antibiotic resistance or a non-host enzyme gene (Allison 2014).

The majority of cloning vectors have been developed for use in *E. coli* cells. In recombinant DNA technology, many cloning vectors are used, depending on the size of the DNA to be cloned and the purpose of the study. Although plasmid and bacteriophage-λ vectors are generally used as vectors for cloning purposes, various cloning vectors such as cosmid vectors, bacterial artificial chromosomes (BAC), yeast artificial chromosomes (YAC), human artificial chromosomes (HAC), and mammalian artificial chromosomes (MAC) are also used (Sözen et al. 2010; Temizkan and Gozukirmizi 2018).

Expression systems

Many proteins produced for research purposes are generally expressed at low concentrations. These proteins can be produced in large quantities with recombinant DNA technology. Recombinant protein production requires cDNA cloning encoding the protein of interest in the expression vector. The choice of an appropriate method to express a recombinant protein is an essential factor in obtaining the desired quantity and quality of the recombinant protein within a reasonable time. Misfolding, deficiency in posttranslational modifications, or inappropriate modifications may occur due to the selection of the wrong expression host. Factors such as the size of the protein and the number of disulfide bonds must be considered when choosing an expression system (Brondyk et al. 2009; Sözen et al. 2010; Temizkan and Gozukirmizi 2018).

Expression vectors, which have all the features of cloning vectors, also carry transcription-initiating promoter and terminating regulatory sequences, which will ensure the desired level of transcription of the gene. Transcription of the gene begins in the promoter region and ends when the terminator sequence is reached. Some promoters are found in expression vectors; lac, tac, phage λPL, and phage T7. Most of the current expression vectors have a ribosome binding site above the start codon, which provides an efficient translation initiation in bacteria. Expression vectors have fewer restriction enzyme cut sites in the multiple cloning region than cloning vectors (Sözen et al. 2010). *E. coli* is one of the most preferred systems as expression systems. Evaluation of recombinant gene expression in *E. coli* takes less than a week. The culture media are inexpensive and relatively easy to understand to increase bioproduction. Recently, *E. coli* systems with T7 promoters are being widely used to express proteins. In addition, *Bacillus subtilis*, *Pichia pastoris*, *Baculovirus*/insect cell, and mammalian expression systems are utilized (Brondyk et al. 2009; Sözen et al. 2010; Temizkan and Gozukirmizi 2018).

Brewer's yeast also reproduces rapidly like bacteria and is inexpensive to produce. They are used in protein expression because they have enzymes to make posttranslational changes. However, since they have many active proteases, protein production can yield reductions. Baculovirus expression vectors in insect cell cultures can also be preferred as protein expression systems because they provide high amounts of protein and allow many of the correct folding and posttranslational changes in proteins. Yeasts have also been used successfully to express proteins. The yeast *Saccharomyces cerevisiae* was the first to be routinely used to express recombinant proteins. However, *Pichia pastoris* has recently become the yeast of choice as it allows higher levels of recombinant protein expression (Cregg et al. 1985; Brondyk et al. 2009; Sözen et al. 2010).

Viruses are one of the most frequently used expression systems in vaccine development studies. Using the baculovirus expression vector system, various proteins used for treatment against many diseases in humans are produced. It is widely used to eliminate drug raw material deficiencies and describe genes belonging to some organisms (Demirbag et al. 1998; Ikonomou et al. 2003; Brondyk et al. 2009; Fernandez-Robledo and Vasta 2010; Temizkan and Gozukirmizi 2018).

Purification of proteins

In order to study the structure and functioning of a protein, it must first be purified. Since proteins differ in some properties such as charge, size and solubility in water, many methods are used to isolate proteins. The two properties mainly utilized in separating proteins are the size of the protein and its binding affinity for a particular binding molecule. Various centrifugation methods, electrophoresis, and liquid chromatography methods are used to separate proteins. The basic principle of centrifugation is the precipitation of particles in a mixed suspension at different rates. There are varieties such as differential centrifugation and velocity-zonal centrifugation. In electrophoresis, which is one of the most commonly used techniques, the molecules in the mixture are separated under the influence of the electric field. SDS-Polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis are commonly used electrophoresis types to separate proteins by molecular weight (Lodish et al. 2011).

Another method used to separate proteins is liquid chromatography. The principle of liquid chromatography is that dissolved molecules interact with a particular solid surface in different ways, such as bonding or separating according to the chemical properties of the surface. If the solution is allowed to flow across the surface, molecules interacting with the surface bind to specific areas, while other molecules do not interact with the surface flow. The sample used in liquid chromatography is placed on spherical particles compressed into a cylindrical column made of glass or plastic. It is then sent down by gravity, pump, or hydrostatic pressure. The fraction leaving the column is collected to analyze the presence of the desired protein. The structure of the filler particles in the

column determines the separation of proteins according to charge, mass, and binding affinity. There are various liquid chromatography methods. These are gel filtration chromatography, ion-exchange chromatography, and affinity chromatography. Among these, affinity chromatography is the most widely used in studies on protozoa (Lodish et al. 2011; Allison 2014).

Some recombinant immunoreactive proteins belonging to protozoa and their usage areas

There are many studies on protozoan diseases using molecular and biotechnological methods, some of which are as follows. Studies detecting immunodominant proteins of *Theileria uilenbergi*, one of the blood protozoa of sheep, stated that these proteins could be used both in developing diagnostic methods and in designing vaccines (Gao et al. 2002; Abdo et al. 2010; Liu et al. 2010). The merozoite antigens of a *Babesia* strain (*Babesia* sp. BQ1-Lintan) genetically similar to *Babesia motasi* in sheep were purified and used in the ELISA test in China (Guan et al. 2010).

Harning et al. (1996) synthesized the SAG1 (surface antigen1) protein, the immunodominant surface antigen of *Toxoplasma gondii*, as a histidine-labeled fusion protein in *E. coli* cells. The recombinant protein was detected by immunoscreening using *T. gondii* specific human IgG and IgM antibodies and mouse monoclonal antibody (S13). These recombinant fusion proteins purified in Ni-chelate column and liquid chromatography were used as antigens in diagnostic methods to detect anti-SAG1 specific IgG and IgM antibodies.

Ikadai et al. (1999) prepared a cDNA expression library from *Babesia caballi* merozoite mRNAs and immunologically screened this library with BC11D, a monoclonal antibody against *B. caballi* merozoite rhoptry protein. As a result, a cDNA fragment encoding a 48 kDa protein of *B. caballi* was cloned and synthesized as a GST-tagged fusion protein in *E. coli* cells by the pGEX4T expression vector. This recombinant protein was used as antigen in the ELISA test. Specific antibodies were determined in the serum of horses infected with *B. caballi*, and no cross-reactions were observed when examined with ELISA using this antigen. As a result, it was stated that this simple and highly sensitive test could be used in the field to diagnose horses infected with *B. caballi*.

Erster et al. (2015) identified the gene encoding *Babesia ovis* surface protein D (BoSPD) from the cDNA library prepared from *B. ovis* merozoites and then cloned this gene in a plasmid vector. They developed a PCR in which this gene was selected as the target gene to detect *B. ovis* in field samples, experimentally infected sheep, and *Rhipicephalus bursa* ticks.

Several genes of *Theileria annulata* have been cloned, sequenced, and recombinant proteins with various names such as TaD, TaSE, TaSP and TamtHSP70 have been expressed (Schnittger et al. 2002; Schneider et al. 2004; Schneider et al. 2007). Among these, the TaSP recombinant protein is suitable for diagnosing tropical theileriosis (Seitzer et al. 2008).

Toxoplasma gondii matrix antigen (MAG1) was cloned, purified, and used in serological techniques by Holec et al. (2007).

Zhou et al. (2007) obtained a cDNA encoding the RAP-1 (rhoptry-associated protein 1) homolog by immunoscreening from a library, they prepared from *Babesia gibsoni* merozoite mRNAs. The whole nucleotide sequence of the gene was found as 1740 bp. Computer analyzes showed that the sequence contains a 1425 bp ORF (open reading frame), which encodes a protein with a molecular weight of 52 kDa. This protein was identified as *Babesia gibsoni* RAP-1 (BgRAP-1) based on sequence similarity. BgRAP-1 gene was expressed in *E. coli* BL21 strain and used as antigen in recombinant BgRAP-1 ELISA assay. It was determined that recombinant BgRAP-1 protein might be useful as a diagnostic antigen for detecting antibodies in dogs infected with *B. gibsoni*.

Pathogen-specific immunoreactive proteins used in the serological diagnosis of *B. ovis* infections in sheep have also been produced. Two immunoreactive proteins were obtained from *B. ovis* by recombinant methods. To detect immunoreactive proteins, a lambda phage cDNA library was constructed from mRNA molecules of *B. ovis* merozoites. As a result of the immunological screening of this library using immune serum, cDNA fragments encode two important proteins named *Babesia ovis*-secreted antigen 1 (BoSA1) and *Babesia ovis*-secreted antigen 2 (BoSA2) were determined. The BoSA1 cDNA fragment consists of an open reading frame of 1137 base pairs, and this open reading frame encodes the BoSA1 protein consisting of 378 amino acids with two internal repeat domains. The length of the BoSA2 cDNA fragment consists of 1161 base pairs and encodes the BoSA2 protein consisting of 385 amino acids with two internal repeat domains. These fragments cloned in pGEX expression vectors were amplified in competent *E. coli* DH5α cells and the proteins encoded by these fragments were synthesized as GST-fusion proteins by IPTG induction. The antigenicity of the proteins purified by glutathione affinity chromatography after synthesis was investigated by Western blot and ELISA methods. The results showed that these recombinant proteins could detect *B. ovis*-specific antibodies in both experimental and natural infected sheep. Therefore, these proteins appear promising as antigens that can be used to develop serological methods for the diagnosis of *B. ovis* infections (Sevinc et al. 2015a; 2015b). In addition, a recombinant protein named ovipain-2 obtained from *B. ovis* seems promising in subunit vaccine and chemotherapeutic drug development studies (Carletti et al. 2016).

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