(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 November 2006 (09.11.2006)

PCT

(10) International Publication Number
WO 2006/117007 A1

(51) International Patent Classification:
C07K 16/44 (2006.01)

(21) International Application Number:
PCT/DK2006/050017

(22) International Filing Date: 2 May 2006 (02.05.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2005 00647 3 May 2005 (03.05.2005) DK

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2006/117007 A1

(54) **Title:** METHODS FOR OBTAINING ANTIGEN SPECIFIC ANTIBODIES

(57) **Abstract:** The present invention relates to methods for obtaining polyclonal antibodies in animals, preferably in chicken. The methods disclosed herein provide tools for obtaining antigen specific antibodies in a faster and more inexpensive way compared to previously known methods.

METHODS FOR OBTAINING ANTIGEN SPECIFIC ANTIBODIES

Technical field of invention

5 The present invention relates to the field of antibodies. In particular, the invention concerns methods for obtaining polyclonal antibodies from avian species and more specifically from chicken (*Gallus gallus domestica*). The present invention also relates to methods for obtaining antigen specific antibodies.

10 Background of the invention

Antibodies are widely used as therapeutic agents, diagnostic tools, research tools, etc.

Polyclonal antibodies are conventionally obtained by immunising a suitable host, usually
15 mammalian with a composition comprising the molecule of interest as an antigen and a suitable adjuvant. Several weeks later, polyclonal antibodies specific to the antigen can be harvested by bleeding the animal and collecting the serum.

It follows that polyclonal antibodies bind different parts of the antigen and individual
20 polyclonal antibodies might likewise bind virtually the same parts of an antigen with different affinities.

Polyclonal antibodies are usually obtained from mammals such as rats, mice, goats, horses, sheep, rabbits, etc. A large amount of experimental animals are used to generate
25 different antibodies – one animal is used per antigen. It follows that the animal will experience discomfort associated with the immunisation and in particular the bleeding procedures. It also follows that it is expensive and laborious to obtain antibodies specific to a large number of different antigens since a similar large amount of animals is required.

30 On one occasion however, the possibility of immunising individual animals with more than one antigen has been discussed in connection with a semi-automated method of hybridoma generation using mice immunised with multiple antigens and a novel antigen microarray assay (Proteomics 5: 4070-4081, 2005 and WO 03089471). In this document, mice were immunised with 10 µg of 5-10 different protein antigens having sizes ranging
35 from about 12 to about 48 kDa in order to solve the bottleneck problem in providing monoclonal antibodies to proteins encoded by the human genome. In the discussion of the scientific paper it is stated that "*It is also possible that increasing the number of immunogens for each animal may ultimately lower the number of positive clones in any one fusion. Indeed, subsequent production runs within our laboratory have shown that five*

antigens per animal are optimal". According to WO 03089471, a major problem associated with immunising with more than one antigen is immuno dominance. In order to circumvent this problem, the animal was injected with several antigen boosters. It thus appears that immunisation with more than one antigen is not recommended, apart from perhaps in special cases involving automated procedures for generating mice monoclonal hybridoma B cell lines, in which case it is recommended to immunise with no more than 5-10 different purified protein antigens. In the patent application it is also suggested that immunisation might be carried out with up to 50 different antigens. However, no specific examples demonstrate that this approach will work with a reasonable expectation of success.

Conventionally obtained antibodies have several other drawbacks in addition to being relatively expensive and time consuming to produce. It may e.g. be difficult to obtain a sufficient response toward mammalian antigens. In general, the large degree of conservation of immunoglobulin structure, immunoglobulin receptor structures, etc. among mammals does in many cases give rise to inaccurate test results when using mammalian antibodies for e.g. detecting mammalian antigens. Also, the problems with protein A/protein G binding, interference with rheumatoid factor, and activation of mammalian complement are often encountered when using mammalian antibodies and mammalian antigens.

In The FASEB Journal 4:2528-2532, 2001 it is disclosed that by immunising a chicken during a 20 day period with a total amount of about 30-300 µg antigen, an immune response could be detected about 2-3 weeks later. It is concluded that the egg yolk is a convenient source of polyclonal antibodies because:
- the quantity of antigen needed is much lower compared to rabbits,
- birds produce antibodies against highly conserved mammalian antigens,
- the use of complete Freunds adjuvant leads to very high and long lasting titers of yolk antibodies, starting as early as 16 days after the first immunisation,
- the purification of antibodies is simple and quick, and a purity of 90% is easily attained by PEG precipitation,
- chicken are inexpensive to keep and easy to handle,
- chicken antibodies are acid and heat resistant and might therefore be orally applied to prevent or to cure intestinal diseases of young animals or humans.

The document does not however, discuss if specific antibodies can be generated in a much faster and much more inexpensive way.

It is thus known that a number of advantages are associated with obtaining antibodies in a non-invasive way from eggs from an immunised chicken rather than collecting blood from an immunised mammal. However, the time required for generating antibodies is not

dramatically reduced when using hen/chicken antibodies. And also, a large amount of birds are used to generate antibodies in cases where a large amount of different antigens are used. The available literature in this field does not provide any suggestions for providing specific antibodies in a dramatically faster and more inexpensive way.

5

In Handbook of Laboratory Animal Science, Second Edition, Vol 1, Jann Hau e.g. it is disclosed that *"For each antigen, there is a dose called the "window of immunogenicity." Too much or too little antigen may induce suppression, sensitization, tolerance, or other unwanted immunomodulation[7]. Very low doses (<1 to 5 µg) are used to induce*
10 *hypersensitivity (allergy),[9,49] and should be avoided in immunization of animals, particularly because booster injections may result in anaphylactic shock in the animals."*
The cited papers are:
[7] B.T Bennett et al., Review of polyclonal antibody production procedures in mammals and poultry, ILAR News, 37, 93, 1995.
15 [9] J. Kollerup et al., Effect of homogenization and pasteurization on the allergenicity of bovine milk analysed by a murine anaphylactic shock model, Clin. Allergy, 17, 5, 449-458, 1987.
[49] J.Hau et al., Reagin production in mice: effect of subcutaneous and oral sensitization with untreated bovine milk and homogenized bovine mik, In Vivo, 3, 271, 1989.

20

It is thus a general presumption in the art that repeated immunizations with low doses of allergen may result in anaphylactic shock and it is not possible to immunise animals with a large amount of antibodies.

25 **Summary of the invention**

The present invention aims at solving these problems by providing a method of obtaining polyclonal antibodies specific to a pool of antigens, wherein the method comprises immunising a vertebrate animal with a pool of antigens, and subsequently collecting
30 polyclonal antibodies.

Antibodies have been raised in animals for decades – using one antigen per animal. According to the present invention however, antigen specific antibodies can surprisingly be obtained by immunising an animal with a large number of antigens. It might thus be
35 concluded that a technical prejudice have been overcome by successfully immunising animals with a large number of antigens.

The invention likewise relates to a method of isolating antigen specific antibodies from an antibody pool, wherein the method comprises immobilising the antigen, contacting the

antibody pool with the immobilized antigen, removing unbound antibody, and recovering antigen specific antibodies.

Normally, affinity chromatography is used for capturing antigen by means of immobilised
5 antibodies. By reversing this principle, the possibility of isolating antigen specific antibodies from an antibody library arises. It follows that the likelihood of isolating polyclonal antibodies specific to any antigen increases with increasing number of different antigens used for immunisation. As a result, a tool for providing specific polyclonal antibodies within a very short time is provided by bypassing the steps of immunisation and waiting for an
10 immune response to occur.

Finally, the present invention relates to the products resulting from the various methods.

Detailed description of the invention

15

In a first aspect, the present invention relates to a method of obtaining polyclonal antibodies comprising antigen specific antibodies, wherein said method comprises the following steps:
(a) immunising a vertebrate animal with a composition comprising: (i) about 15 or more
20 different peptide antigens, said peptides being conjugated with a carrier and having a length of at least 6 amino acids, and (ii) an adjuvant,
(b) collecting a pool of polyclonal antibodies from the animal, wherein a fraction of about 1% or more show reactivity towards the antigens used for immunisation.

25 The principle of immunising an animal, preferably a bird of the gallinacean type, and most preferably of the species *Gallus gallus domestica* with a large amount of different antigens is a principle that provides tools for obtaining polyclonal antibodies in a much more fast and inexpensive way than previously known methods. The "antibody library" can subsequently be used for isolating polyclonal antibodies with a desired specificity. After
30 having isolated specific polyclonal antibodies, the antibody library can be stored for later usage and the library can thus be "recycled" in an (in theory) indefinite number of times. It follows, that it is much faster to isolate antigen specific antibodies from an antibody library which in theory comprises antibodies that have antibody binding capability to almost any antigen – rather than immunising the animal and waiting several weeks for a specific
35 antibody response.

A preferred way of obtaining an antibody library is by immunising with a peptide library obtainable by synthesizing peptides with a length of at least 6 amino acids. Likewise, the peptide antigen may also be a mix of peptides with different lengths.

The hen is ideal because it can produce about 1500 mg antibodies per month (about 50-100 mg antibodies per egg (AOEA 24: 925-934, 1996). In fact, antibodies can be obtained in a non-invasive manner from the chicken during the entire egg-laying period. Usually, about 2-10% of the antibodies are antigen specific (AOEA 24: 925-934, 1996). The percentage of antigen specific antibodies may be even higher as a response to immunisation with a multitude of antigens.

Ideally, the antibodies are pooled and stored until a time where it is desired to isolate antigen specific antibodies. Antibodies can be stored at subzero degrees (e.g. liquid nitrogen, -70°C, or -20°C). However, if the library is used for frequent isolation of antigen specific antibodies, it may be preferable to avoid freezing by storing the library at temperatures in a range of about 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10°C. Even though antibodies tend to be stable, repeated freeze/thaw circles may result in some degree of protein denaturation of the antibodies.

Antibodies according to the invention may furthermore be stored in the presence of one or more additives such as: antibiotics, anti-fungal compounds, antifreeze (e.g. glycerol), and buffer solutions. A preferred additive is NaN_3 – preferably 0,1% NaN_3 in PBS. Antibodies may even be stored at room temperature, preferably in the presence of preservatives, for relatively short period of times such as e.g. two weeks or less, preferably one week or less. This might be convenient e.g. in case of transport of antibodies via shipping, mailing, etc.

In a preferred embodiment, antibodies are collected from the animal after a period of at least 14 days, since this period of time is usually required to induce a detectable immune response in e.g. a chicken subsequent to immunisation.

The present invention furthermore relates to antibodies obtainable by the methods herein.

Another central aspect of the present invention relates to the use of antibody libraries being specific to a multitude of antigens. In particular, the present invention thus relates to a method of isolating antigen specific antibodies, wherein the method comprises the following steps:
- immobilising the antigen,
- contacting the antibody library with the immobilized antigen under conditions suitable for an antigen:antibody binding to take place,
- removing unbound antibody, and
- recovering antigen specific antibodies.

An antibody library obtained after immunising an animal with a peptide library generated by random synthesis thus provides a unique possibility of isolating antibodies with specificity to an antigen within a very short period of time – in theory within few hours.

5 Various methods of immobilising the antigen can be employed in connection with isolation of antigen specific antibodies. A preferred solid phase is CnBr carrier. A preferred coupling is Epoxy coupling since this approach is relatively cheap. Ultrasound sonification or acidic conditions may e.g. be used for breaking the antigen:antibody binding in order to recover antigen specific antibodies. In general, various methods of coupling proteins to a carrier
10 and subsequently breaking protein:protein interactions are well known in the art.

Finally, the present invention relates to antibodies obtainable by the methods herein.

Definitions

15

Peptide antigen: Peptides according to the present invention are defined as peptides with a length of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids. Peptides according to the present invention may also have lengths of about 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 225, 250, 275, 300,
20 325, 350, 375, 400, 425, 450, 475, 500, 525, 550, 575, 600 amino acids or more. The peptides used as antigens according to the invention can be a mix of peptides of different lengths, i.e. there may be short peptides present with a length as short as 6 amino acids as well as longer peptides. Some or all of the peptides may also be in form of proteins being either denatured or in their naturally folded form. It follows that proteins can be
25 obtained from either a natural source or they can be recombinantly produced and subsequently purified. The peptides may in addition comprise any form of modifications such as e.g. glycosylation, lipid attachment, hydroxylation, disulfide bonding, phosphorylation, etc. The peptides may also originate from a proteolytical digest of peptides and/or proteins. Antigens according to the present invention are in other words of
30 peptide origin with a length at or above 6 amino acids, and may be derived either from natural sources, produced by recombinant methods, or synthesized artificially – or any mixtures thereof. It is however important that the peptides used for immunisation in connection with the present invention are substantially free from e.g. contaminating cellular debris. Thus, the present invention does not relate to immunisation with antigens
35 derived from cellular material including cell membranes, nucleic acids, etc.

The amount of different peptide antigens used for immunisation of the animal is at least 15. As the aim of the present invention is to provide tools for rapidly isolating antigen specific antibodies, it is advantageous to immunise with even more antigens, as the

likelihood thus increases of obtaining antibodies with specificity to most antigens. It is therefore preferable to immunise with at least about 25, 50, 100, 150, 200, 250, 500, 1000, 5000, 10000, 50000, 100000, 500000, 1000000, or 5000000 different antigens. It is most preferable to immunise with a synthetically synthesized peptide library comprising, in theory, about 20^6 or more different peptide combinations.

Peptide library: A preferred way of carrying out the present invention is to synthesize a peptide library with peptides of similar lengths covering the possible amino acid combinations of the 20 naturally occurring amino acids. Peptide synthesis may take place in solution or on a solid phase. A peptide library with peptides of a length of e.g. 6 naturally occurring amino acids will thus in theory have 20^6 different ways of combining the amino acids, and thus up to 20^6 different peptides. The peptide library may also be a library comprising e.g. a specific fraction of the amino acid sequence from a number of different proteins with known sequences. The peptides must have lengths of at least 6 amino acids in order to be able to bind to the hyper variable loops of the Fab fragment of the antibody. It follows that peptide libraries may also be prepared using less than 20 amino acids – in which case of course the theoretical number of different combinations will vary accordingly.

Carrier: Conjugation to a carrier is important because peptides are small molecules that alone do not tend to be immunogenic, thus possibly eliciting a weak immune response. The carrier contains many epitopes that stimulate T-helper cells, which help induce the B-cell response. Many different carriers can be used for coupling to synthetic peptides. The most commonly selected carriers are keyhole limpet hemacyanin (KLH) and bovine serum albumin (BSA). The higher immunogenicity of KLH often makes it the preferred choice. Another advantage of choosing KLH over BSA is that BSA is used as a blocking agent in many experimental assays. Because antisera raised against peptides conjugated to BSA will also contain antibodies to BSA, false positives may result. Although KLH is large and immunogenic, it may precipitate during cross-linking, making it difficult to handle in some cases. Ovalbumin (OVA) is another useful carrier protein. It is a good choice as a second carrier protein when verifying whether antibodies are specific for the peptide alone and not the carrier. Rabbit Serum Albumin (RSA) may be used when the antibody response to the carrier protein must be kept to a minimum. Rabbits immunised with RSA conjugate are less likely to raise antibodies to the carrier, as the RSA is recognized as "self." If the RSA conjugate were injected into another host, the protein would not be recognized as self. The same principle, of course, applies when immunising with chicken-derived carriers in chicken. It is important to recognize that the immune system reacts to the peptide-protein carrier as a whole and that there will be a portion of response directed against the conjugated peptide as well as the linker and the carrier. When screening by ELISA it is

advisable to use a peptide conjugate prepared using a different carrier. This is not necessary if performing ELISA assays where the plates are coated directly with unconjugated peptide.

5 Suitable carriers will thus be of protein origin in most cases. In some cases however, the carrier may be of non-protein origin such as e.g. polymers comprising carbohydrates, etc.

It follows that it may not be necessary to conjugate relatively large peptides with a carrier in order to obtain an immune response. Peptides with a length of at least about 100 amino
10 acids and more preferably with a length of at least about 150 amino acids may not need carrier conjugation in order to function as immunogens in the present invention. It is however advisable to conjugate all peptides with carrier molecules without regard to the size.

15 Immunisation of the animal may be carried out with or without pharmaceutical carriers. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Additionally, these
20 carriers may function as immunostimulating agents ("adjuvants"). Immunisation of the animal may be carried out with adjuvants and/or pharmaceutical carriers. An adjuvant is any substance that enhances the immune response to an antigen with which it is mixed. The antigen may also be mixed with two or more different adjuvants prior to immunisation. Examples of commonly used adjuvants comprise: Immunostimulatory oil
25 emulsions (for example, water-in-oil, oil-in-water, water-in-oil-in-water such as e.g. Freund's incomplete adjuvant such as Montainde®, Specol, mineral salts such e.g. as $Al(OH)_3$, $AlPO_4$, microbial products, Saponins such as Qual A, synthetic products, as well as adjuvant formulations, and immune stimulatory complexes (ISCOMs). A list of other commonly used adjuvants is disclosed on pages 6-8 in WO 03089471, the list being hereby
30 incorporated by reference. The most preferred adjuvant according to the present invention is Specol since a fast immune response is initiated. Normally, antibodies can be harvested three to six weeks after immunisation in e.g. hen, but when using Specol as an adjuvant, antibodies may be harvested after only about two weeks.

35 Adjuvants are generally included in the immunogenic compositions in an amount according to the instructions of the manufacturer. If the adjuvant is Specol an amount of e.g. 5, 10, 15, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 500, 600, 700, 800, 900, 1000, 1500, or 2000 µl may be added to the immunogenic composition. Preferably, the adjuvant is mixed with water or buffer in an amount of about 1:1.

Immunisation: Antigen may be introduced into the animal by any suitable means. Preferably, the method of introduction involves injection. The animals may be immunised with the antigens intrasplenically, intravenously, intraperitoneally, intradermally or
5 subcutaneously or by any other suitable means. For practical and economical reasons, chickens kept under field conditions are vaccinated intramusculary in the breast muscle. In the laboratory, chickens can also be vaccinated subcutaneously in the neck.

The total number of immunisations will depend upon the type and dose of the antigen, as
10 well as on the particular adjuvant employed. In a preferred embodiment, at least two immunisations should be given. If the antibody titres begin to decrease, booster immunisations can be given.

The total amount of peptide employed for each immunisation may likewise vary. Amounts
15 of about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 µg are preferably used in a single immunisation. But amounts of about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, 200, 250, or 500 mg may also be used in a single immunisation.
20

Vertebrate animal: A vertebrate animal is herein to be understood as an animal from the vertebrate phylae. Preferred animals are animals from either the mammalian or the avian orders. Avian animals are particularly preferred. Birds from the Galliformes order are most preferred. Birds used for immunisation should have an age of at least 17 weeks, preferably
25 at least 19 weeks.

Antibodies: Antibody molecules are plasma proteins that bind specifically to particular molecules known as antigens. Antibody molecules are produced by B lymphocytes in response to immunisation with antigen. They are the specific molecules of the humoral
30 immune response that bind to and neutralize pathogens or prepare them for uptake and destruction by phagocytes. Each antibody molecule has a unique structure that allows it to bind its specific antigen, but all antibodies have the same overall structure and are known collectively as immunoglobulins. The body can make an almost unlimited variety of different antibodies, each B lymphocyte being genetically programmed early in its
35 development to produce antibodies of a single specificity. A pool of antibodies according to the present invention can be obtained by immunising a single animal with a randomly generated peptide library. A randomly generated peptide library will in general stimulate an antibody response comprising antibodies with binding capability of (in theory) any antigen.

Antibody specificity toward an antigen: According to the present invention an antibody can be termed antigen specific if e.g. antigen:antibody binding can be demonstrated in an assay as disclosed in Example 4, where antigen is linked to a carrier in the presence of PBS and antibodies are likewise suspended in a PBS buffer.

5

According to the present invention, at least about 1% of the antibodies obtained subsequent to immunisation show specificity to the antigens used for immunisation. If less than 1% of the antibodies show specificity to the antigens, antibodies may e.g. have been harvested too soon after immunisation, insufficient amounts of antigen have been
10 employed, or inefficient amounts of adjuvants have been employed. More preferably, at least about 2, 3, 4, or 5% of the antibodies are antigens specific. Most preferably, at least about 6, 7, 8, 9, or 10% of the antibodies are specific. And most preferably about 20-30% of the antibodies are antigen specific.

15 Antigen: Antigens are molecules that react with antibodies.

Conditions suitable for an antigen:antibody binding to take place: such conditions are well known in the art. In the Examples, PBS buffer is employed.

20 Elution: By using different elution conditions, the method allows an approximate quantification of the binding affinity of the monoclonal antibody for its binding partner. Elution agents that may be used include chaotropic agents such as guanidine hydrochloride or urea at concentrations between 10 µM and 8 M or ethylene glycol in an aqueous solution of 0.01% to 100% w/v. Elutions may also be carried out using aqueous or non-aqueous
25 solutions of glycine. Elutions may be carried out using aqueous or non aqueous solutions of triethylamine between 1 µM and a saturated solution, preferably 100 mM, at a pH of between pH 8 and pH 13, preferably pH 11.5.

Galliformes: The order of birds that includes grouse, ptarmigan, capercaillie, partridges,
30 pheasants, quails, turkeys and peacocks. These are mainly grain-eating, heavy-bodied, ground-nesting birds, capable of only short, rapid flights. The cocks are usually more colourful than the hens. A preferred bird according to the present invention is a hen (*Gallus gallus domestica*) either in the adult stage or as a chicken at the age of at least 17-19 weeks.

35

Bursa fabricii: A thymuslike lymphoid gland in birds that is an outgrowth of the cloaca and the site of B cell maturation.

EXAMPLES

Example 1

5 Synthesis of a peptide library (length of peptides: 6 amino acids)

A library of peptides with a length of 6 amino acids was synthesized using standard methods. The 20 naturally occurring amino acids were employed in each reaction and the library thus synthesized comprises (in theory) 20^6 different peptides.

10

Example 2

Conjugation of peptides with carrier

15 The peptide sequence must contain at least one cysteine residue in order for the cross linking agent (MBS) to efficiently couple the peptides to the carrier protein Keyhole Limpet Hemocyanin (KLH). In this Example, a cysteine residue was added to the peptides as the seventh residue before carrier conjugation. It should be noted that it is also possible to conjugate via the amino terminal end when using MBS as a coupling agent.

20

Sephadex G-25 fine beads were swelled overnight in 5 ml buffer (0.1 M NaH_2PO_4 pH 6.0)/g of dry beads at room temperature. The swelled resin was washed 4 times using 0.1 M NaH_2PO_4 pH 6.0 and poured into a 130 x 20 mm column. The column was subsequently equilibrated with 15 ml of 0.1 M NaH_2PO_4 pH 6.0 and the resin was allowed to settle.

25

4 mg KLH was dissolved in 0.3 ml of 0.1 M NaH_2PO_4 pH 6.0. 1 mg m-Maleimidobenzoyl-N-Hydroxysuccinimide ester (MBS) in 150 µl of Dimethylformamide (DMF) was added drop-wise to the KLH solution under stirring. The KLH/DMF mixture was incubated at room temperature for 30 min on a rotating mixer.

30

1 ml of 0.1 M NaH_2PO_4 pH 6.0 was added to the KLH-MBS conjugate followed by thorough mixing. The mixture was subsequently loaded onto the Sephadex G-25 column. 0.1 M NaH_2PO_4 pH 6.0 was used as column buffer.

35 1 ml fractions were collected by gravity flow in 1.5 ml tubes. The fractions comprising the KLH-MBS conjugate (monitored at 280 nm) were pooled in a 15 ml conical tube.

200 µl of Dimethylformamide (DMF), 1 ml of peak fractions at 280 nm pH 6.0, and 5 mg of the cysteine-containing peptide were mixed.

The peptide solution was mixed with the KLH-MBS conjugate and incubated for 12 to 16 hr at room temperature on a rotating mixing wheel.

5 The KLH-MBS-peptide conjugate was dialyzed for 24 hr at 4°C against 1 liter of PBS including two changes of buffer. Molecular weight cut off of the dialysis tube was 3,500.

Example 3

10 Generation of an immunologic library - Immunisation of a chicken with peptides

20 and 35 weeks old fowls of the "white Italian" race were immunized on day 0, 10, 20, 100 and 200. Blood samples and eggs were taken on day 0, 7, 14, 21, 28, 35, 42, 49 and antibody concentration (IgY) was measured on day 7, 14, 21, and 28 using ELISA
15 techniques. Antibody titers were increasing from about day 21 and onwards.

The composition used for each immunisation: 10 µg KLH-peptide dissolved in 0.01 M NaCl buffer (pH 7.2) to a volume of 500µl and 500 µl Specol to a total volume of 1 ml.

20 IgY purification

Yolk was separated from egg white. The yolk was punctured and dissolved in 1:10 weight/volume 2xH_2O. The mixture was frozen and thawed. Ammonium sulfate was added to the thawed yolk to 25 % saturation, and the mixture was incubated at 20°C for 20 min.
25 Subsequently, the mixture was centrifuged in 30 min at 2500 x g. The supernatant was harvested, ammonium sulfate was added to 40 % saturation, and the mixture was incubated at 20°C for 20 min. The mixture was centrifuged in 30 min at 2500 x g. Pellet comprising IgY was dissolved in 1 ml PBS/0.1% NaN_3 and kept at -20°C.

30 **Example 4**

Isolation of antigen specific antibodies

One gram of cyanogen bromide-activated sepharose 4B (Pharmacia) was swollen according
35 to manufacturers instructions to yield 3.5 ml of resin.

2.5 mg peptide was dissolved in 0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3 and added to the swollen sepharose. The peptide:resin mixture was mixed for 2 hours at room temperature and centrifuged at 40 x g for 5 min. The resin was subsequently incubated with blocking

buffer (0.2 M Glycine, pH 8.0) at 4°C for 16 h. The peptide:resin was then poured into a column and washed 5 x with 0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3 followed by 5 ml 0.5 M NaCl, 0.1 M ammonium acetate, pH 4.0.

5 After rinsing the column with 50 ml PBS, 8 ml IgY obtained in Example 3 was mixed with 42 ml PBS and loaded overnight at 4°C at a flow rate of 30 µl/min. The column was subsequently washed with 50 ml PBS. The antibodies that did not bind to the column were dialyzed for 24 hr against 1 liter of PBS with 2 buffer changes 4°C. Cut off: 3,500. After dialyzation, antibodies can be returned to library to be used for another occasion.

10

Antigen specific antibodies were eluted from the column with 0.15 M NaCl, 0.2 M glycine, pH 2.2. The eluate was neutralized with 1 M Tris-HCL pH 8.0 and stored at 4°C with 0.01 % NaN_3.

15 Affinity of antigen specific antibodies

To measure the antibody affinity, purified antibody is reloaded on column. Instead of eluting with pH 2.2 a range from pH 8.0 to pH 1.0 is used. The amount of antibody released at a specific pH is measured at 280 nm. The absorbents peak of the pH range

20 indicates its affinity.

Example 5

Library immunisation experiment:

25

10,000 peptides with a length of 15 amino acids were synthesized in a random manner in order to obtain a peptide library as described above. Four control peptides of the same length, known to be immunogenic in hen (C, Ec, Sf-2, Sf-3), were synthesized as well. Cystein residues were added at the C-terminal end. The peptides were subsequently

30 conjugated with a carrier (KLH).

2 mg peptide library conjugated with carrier was mixed with 0,2 µg of each control peptide along with 2 ml adjuvant (1 ml Specol + 1 ml water). A 19 weeks old Isababcock hybrid hen was immunized with the immunogenic composition. The hen was reimmunised on day

35 10 after the first immunization.

Blood samples from the hen were collected before (22 March) and after immunisation (4 April and 18 April). Diluted samples (1:100) were tested with ELISA. The secondary HRP antibodies were diluted 1:10,000. In the ELISA-tests, each well was precoated with 100 ng

peptide or peptide library. Controls were performed wherein the wells were not precoated with any protein or peptide. The results are shown in the table:

ELISA Results

Date	KLH	library	library	C	C	Ec	Ec	Sf-2	Sf-2	Sf-3	Sf-3
22/3	0	0	0	0	0	0	0	0	0	0	0
4/4	0	0	0	0	0	0	0	0	0	0	0
18/4	2,52	1,84	1,90	0,05	0,04	0,04	0,04	0.07	0,07	0.01	0,01

5

Table 1: Induction of immune response in a hen to selected target antigens. The hen was immunised with a large amount of different peptides. Control samples were blood samples collected before immunisation. The results shown in the table are reproducible.

10 It thus appears that contrary to the general presumptions in the art, it is possible to immunise with 10,000 different peptides AND obtaining specific antibodies. It is thus possible to produce a large amount of different antibodies in one animal and thus exploiting the ability of the hen to produce large amounts of antibodies which can be collected from the eggs. It is furthermore demonstrated that immunisation with small
15 amounts of antigen is possible without inducing anaphylactic shock in the animal.

20

Claims

1. A method of obtaining polyclonal antibodies comprising antigen specific antibodies, wherein said method comprises the following steps:
5 (a) immunising a vertebrate animal with a composition comprising: (i) about 15 or more different peptide antigens, said peptides being conjugated with a carrier and having a length of at least 6 amino acids, and (ii) an adjuvant,
(b) collecting polyclonal antibodies from the animal, wherein a fraction of about 1% or more shows reactivity towards the antigens used for immunisation.
10

2. A method according to claim 1, wherein the vertebrate animal is of the species *Gallus gallus domestica*.

3. A method according to anyone of claims 1 or 2, wherein the animal is reimmunised.
15

4. A method according to any one of claims 1-3, wherein the antibodies are collected from the animal after a period of at least 14 days.

5. A method according to any one of claims 1-4, wherein the adjuvant is Specol.
20

6. A method according to any one of claims 1-5, wherein the carrier is KLH.

7. A method according to any one of claims 1-6, wherein the peptide antigens is a peptide library obtainable by randomly synthesizing peptides with a length of at least 6 amino
25 acids.

8. A method according to claim 7, wherein the peptide antigen is a mix of peptides with different lengths.

30 9. Antibodies obtainable by any of the methods according to claims 1-8.

10. A method of isolating antigen specific antibodies from an antibody pool according to claim 9, wherein the method comprises the following steps:
- immobilising the antigen,
35 - contacting the antibody pool with the immobilized antigen under conditions suitable for an antigen:antibody binding to take place,
- removing unbound antibody, and
- recovering antigen specific antibodies.

11. A method according to claim 10, wherein the antigen specific antibodies are recovered using acidic conditions.

12. A method according to any one of claims 10-11, wherein the antigen is immobilized on
5 a CnBr carrier.

13. A method according to any one of claims 1-8, or 10-12, wherein the antibodies are stored in presence of one or more additives selected from the group consisting of: antibiotics, anti-fungal compounds, and buffer.

10

14. A method according to any one of claims 1-8, or 10-13, wherein the antibodies are stored at a temperature of about 0-10°C.

15. Antibodies obtainable by a method according to any one of claims 1-8, or 10-14.

15

16. Use of antibodies according to claim 9 in a method according to any one of claims 10-15.

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2006/050017

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 03/089471 A (EUROPEAN MOLECULAR BIOLOGY LABORATORY; SAWYER, ALAN, MICHAEL; DE MASI,) 30 October 2003 (2003-10-30) the whole document	1,3,4
X	US 2002/168684 A1 (COMB MICHAEL J ET AL) 14 November 2002 (2002-11-14) the whole document	1,3,4,6, 7
X	COOK CARRIE L ET AL: "Simple purification methods for an alphagalactose-specific antibody from chicken eggs" JOURNAL OF BIOSCIENCE AND BIOENGINEERING, vol. 91, no. 3, March 2001 (2001-03), pages 305-310, XP002395979 ISSN: 1389-1723 the whole document	10,11

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 August 2006	08/09/2006

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	HANLY ET AL.: "Review of polyclonal antibody production procedures in mammals and poultry" ILAR JOURNAL, vol. 37, 1995, page 93-118, XP001247288 page 99, column 100 -----	1-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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