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(54) **Title**: METHOD OF PURIFYING ANTI-IDIOTYPIC ANTIBODIES

(57) **Abstract**: The present invention discloses a method for purifying anti-idiotypic antibodies using a series of purification via chromatographic columns, wherein the columns comprise targeted antibody coupled affigel hydrazide column; human IgG coupled affigel hydrazide column; and human IgG coupled NHS activated sepharose. Further, the anti-idiotypic antibodies obtained from the said method contains higher specificity and negligible cross reactivity.

METHOD OF PURIFYING ANTI-IDIOTYPIC ANTIBODIES

RELATED APPLICATION

This application is related to and takes priority from Indian Provisional Application 2922/CHE/2013 filed on 16th June, 2014 and is herein incorporated in its entirety.

BACKGROUND

The present invention relates to a method for purifying antibodies, more particularly anti-idiotypic antibodies which are raised against a targeted antibody.

Monoclonal antibodies are highly successful in treatment of various diseases including oncolological and autoimmune diseases. These antibodies bind to target site through a unique part of variable portion known as “antigenic determinants” or “complementary determining region (CDR)” or “idiotopes”. Antibodies raised against idiotopes are known as anti-idiotypic antibodies.

Anti-idiotypic antibodies specifically bind to antigenic determinants of the target antibody against which they have been raised. Due to the specific binding nature of the anti-idiotypic antibodies, they have wide variety of applications in various assays, as control or in detecting the target antibody. Other applications of these anti-idiotypic antibodies include use in pre-clinical research, pharmacokinetic studies, immunogenicity assays, as positive controls in immunogenicity assays and ligand binding or cell based neutralizing assays, and in antibody blocking assays etc.

Generally, anti-idiotypic antibodies are raised by injecting the variable portion of the targeted antibody in an animal. However purification of the generated anti-idiotypic antibodies from the serum collected from the animal or serum of any origin containing anti-idiotype antibodies is cumbersome as the serum contains various endogenous immunoglobulins and antibodies against other portions of targeted antibody other than CDR.

Purification of anti-idiotypic antibody is generally performed by affinity chromatography, wherein selection of the column depends on the nature of the anti-idiotypic antibody. During purification of anti-idiotypic antibodies, columns are selected

based on the chemistry of conjugation of an affinity molecule with the column matrix which in turn determines the availability of the conjugated molecule. Further, such availability of conjugated molecule helps in better purification of specific anti-idiotypic antibodies. Apart from selection of columns, buffers employed in purification process also play an important role. Hence it is essential to select appropriate columns and optimize various parameters to obtain highly specific anti-idiotypic antibody.

Hence the primary object of the present invention is to provide a method for purification of anti-idiotypic antibodies raised against a targeted antibody from a serum sample. Another objective of the invention is to provide purified anti-idiotypic antibody with higher specificity and negligible cross-reactivity.

SUMMARY

The present invention discloses a method for purification of anti-idiotypic antibody from serum containing anti-idiotype antibodies. The method comprises performing a series of affinity chromatography for purification of said anti-idiotypic antibody, wherein the method uses columns comprising a targeted antibody coupled affigel hydrazide column; a human IgG coupled affigel hydrazide column; and a human IgG coupled N-hydroxy succinimide (NHS) activated sepharose.

Furthermore, the series of affinity chromatography columns for purification, as disclosed in the present invention provides a simple and broadly applicable purification process to obtain highly specific anti-idiotypic antibodies for use in further applications.

DETAILED DESCRIPTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains to.

The present invention discloses a method of purifying anti-idiotypic antibodies from serum containing anti-idiotype antibodies using affinity chromatography, wherein sequential purification steps are performed in columns comprising targeted antibody coupled affigel hydrazide column, human IgG coupled affigel hydrazide column and

human IgG coupled NHS activated sepharose to obtain highly specific anti-idiotypic antibodies.

The availability of the coupled antibody in a column to purify anti-idiotypic antibody depends on the chemistry involved in conjugation. In case of antibody coupled to affigel hydrazide, conjugation of said antibody to column occurs specifically through carbonyl groups of oxidized carbohydrates in the Fc portion of the antibody. The said carbonyl groups conjugate to the hydrazide moiety of the affigel column and results in formation of stable covalent hydrazone bonds.

In the said column, due to the binding of the Fc portion, the Fab portion of the conjugated antibody (i.e; targeted antibody or human IgG) is available to bind with the targeted anti-idiotypic antibody in the human sera. Hence all the specific antibodies either against Fab of targeted antibody or human IgG will bind to the column and the rest will get eluted in the flow through.

Conjugation in case of antibody coupled to NHS activated sepharose, occurs through formation of amide bonds between lysine ε-amine groups of the targeted antibody and N-hydroxy succinimide of the sepharose column. Hence various portions of the conjugated antibody molecule is available to anti-idiotypic antibodies present in the sample.

The present invention discloses a method for purification of anti-idiotypic antibodies against CDR portions of the targeted antibody, wherein the serum containing the said anti-idiotypic antibody is incubated with targeted antibody coupled affigel column. In this process, the anti-idiotypic antibodies in the said serum bind to the variable portion of the targeted antibody coupled to affigel hydrazide solid support. The bound anti-idiotypic antibodies are eluted from the column using an acidic buffer.

The eluted anti-idiotypic antibody from the above process is then incubated with human IgG coupled affigel hydrazide column. The anti-idiotypic antibodies which are raised against the CDR portions of the targeted antibody does not bind to the non-specific human IgG coupled affigel hydrazide column. The specific anti-idiotypic antibodies are collected in flow-through using a buffer with neutral pH.

The collected flow through from the human IgG coupled affigel hydrazide column, is further incubated with the human IgG coupled NHS activated sepahrose column. Antibodies that are raised against various portions including framework regions of targeted antibody other than antibodies against CDR portions if any present in the serum binds to the said column. Further, the anti-idiotypic antibodies which are raised specifically against the targeted antibodies does not bind to the column and are collected as a flow through using a buffer with neutral pH.

The anti-idiotypic antibody purification as disclosed in the present invention uses three combinations of sequential purification using said columns where in one of the combination comprises purification *via* targeted antibody coupled affigel hydrazide column followed by human IgG coupled affigel hydrazide column and followed by human IgG coupled NHS activated sepharose column.

Another combination for purification of anti-idiotypic antibodies from sera is performed by incubating the said serum with human IgG coupled affigel hydrazide column followed by targeted antibody coupled affigel hydrazide column and followed by human IgG coupled NHS activated sepharose column.

In addition, purification of anti-idiotypic antibody from sera is performed by incubating the said serum with human IgG coupled NHS activated sepharose column followed by targeted antibody coupled affigel hydrazide column and followed by human IgG coupled affigel hydrazide column.

The purification method wherein, the sera containing anti-idiotypic antibody is incubated with antibody coupled affigel hydrazide column; followed by human IgG coupled affigel hydrazide column; and followed by human IgG coupled NHS activated sepharose column yielded highly specific anti-idiotypic antibodies.

Various embodiments of the disclosed invention provides a method of purifying anti-idiotypic antibodies raised against a targeted antibody in an animal.

In one embodiment the invention discloses a method of purifying highly specific anti-idiotypic antibodies raised against a targeted antibody from serum/composition, comprising;

a) loading the serum containing anti-idiotypic antibody on a targeted antibody coupled affigel matrix, followed by eluting the said bound anti-idiotypic antibodies with an acidic buffer followed by neutralization;

b) loading the neutralized elute obtained in step a) onto a human IgG-coupled affigel matrix followed by collecting the flow through containing anti-idiotypic antibodies and,

c) loading the collected flow through obtained in step b) onto a human IgG coupled NHS activated sepharose matrix followed by collecting the flow through containing purified anti-idiotypic antibody,

wherein the purified anti-idiotypic antibody, obtained by the method of the present invention is highly specific, and contains negligible cross reactivity with other antibodies.

In another embodiment of the invention discloses a method of purifying highly specific anti-idiotypic antibodies raised against a targeted antibody from serum/composition, comprising;

a) loading the serum containing anti-idiotypic antibody on a human IgG coupled affigel matrix, followed by collecting the flow through containing anti-idiotypic antibodies;

b) loading the collected flow through obtained in step a) onto a targeted antibody-coupled affigel matrix followed by eluting the said bound anti-idiotypic antibodies with an acidic buffer followed by neutralization and,

c) loading the neutralized elute obtained in step b) onto a human IgG coupled NHS activated sepharose matrix followed by collecting the flow through containing purified anti-idiotypic antibody.

In yet another embodiment of the invention discloses a method of purifying highly specific anti-idiotypic antibodies raised against a targeted antibody from serum/composition, comprising;

a) loading the serum containing anti-idiotypic antibody on a human IgG coupled NHS activated sepharose matrix, followed by collecting the flow through containing anti-idiotypic antibodies;

b) loading the collected flow through obtained in step a) onto a targeted antibody-coupled affigel matrix followed by eluting the said bound anti-idiotypic antibodies with an acidic buffer followed by neutralization and,

c) loading the neutralized elute obtained in step b) onto a human IgG coupled affigel matrix followed by collecting the flow through containing purified anti-idiotypic antibody.

In another embodiment, the target antibody is selected from any of the groups like chimeric, humanized and human therapeutic antibodies such as rituximab, trastuzumab, bevacizumab, cetuximab, infliximab, adalimumab etc.

In another embodiment, cross reactivity of the purified anti-idiotypic antibody is evaluated with the targeted antibody and Human IgG wherein the S/N ratio is obtained.

DEFINITIONS

The term “idiotype” used here in the invention refers to a unique part in variable portion of an antibody, with which it binds to an antigen or a target site.

The term "anti-idiotypic" or "anti-idiotypic antibody" refers to an antibody raised against a targeted antibody which specifically binds to an idiotope of the targeted antibody.

“Targeted antibody” used here in the invention refers to a therapeutic monoclonal antibody.

"Specificity" is measured in terms of S/N ratios, which are the ratio between mean absorbance of an anti-idiotypic antibody bind to the targeted antibody (S) and mean absorbance of an anti-idiotypic antibody bind to human IgG.

"Cross-reactivity" refers to non-specific binding of anti-idiotypic antibody to other antibodies other than the targeted antibody against which it has been raised.

Certain specific aspects and embodiments of the invention are more fully described by reference to the following examples. However, these examples should not be construed as limiting the scope of the invention in any manner.

Example 1

During the development of a method for purifying anti-idiotypic antibodies raised against rituximab various parameters were optimized. One such important parameter was digestion of rituximab and obtaining purified $F(ab)_2$ fragments of rituximab and immunization of animal with various boosting doses to get desired titer of anti-idiotypic antibodies.

Another parameter was selection of columns for conjugation of various molecules, for which affigel hydrazide and NHS activated sepharose solid supports were selected. Rituximab was coupled to affigel hydrazide, human IgG was coupled to both columns (i.e;NHS activated sepharose and affigel hydrazide).

Immunization of animal to generate anti-idioypic antibodies:

To generate, anti-idiotypic antibodies against rituximab in rabbit, first rituximab was digested with papain to obtain $F(ab)_2$ fragments followed by purifying the $F(ab)_2$ fragment using MabSelect™ Sure resin solid support to remove undigested rituximab and strong ion exchange chromatography (Hi-Trap sepharose column) to remove other contaminants. Further, purified fragments were mixed with Fraund's complete and Fraund's incomplete adjuvants to prepare primary and booster doses for immunization of animals.

Prior to immunization, pre dose serum was collected from the animal followed by, primary immunization with an emulsion containing equal volumes of purified $F(ab)_2$ fragments and Fraund's complete adjuvant. Subsequent immunizations was followed with booster doses containing equal volumes of $F(ab)_2$ fragments and Fraud's incomplete adjuvant on day 14^{th}, 28^{th} and 42^{nd} to obtain desired antibody titer. Once desired antibody titer was achieved, blood was withdrawn from the animal to obtain immune serum from the collected blood. Further, the obtained serum was subjected for purification of anti-idiotypic antibodies.

Purification of anti-idiotypic antibody:

Three different columns were employed for purification of anti-idiotypic antibodies and these columns include rituximab coupled affigel hydrazide solid support, human IgG coupled solid support and human IgG coupled NHS activated sepharose. Human IgG columns were employed for removing anti-human IgG specific antibodies. Coupling of the respective targeted molecules to different columns were performed as per vendor recommendations. Prior to the employment of columns during purification, all columns were washed and equilibrated with phosphate buffer saline (PBS) solution with pH 7.2 at room temperature.

4 ml of serum collected from rabbits was mixed with an equal volume of PBS and loaded on to rituximab coupled affigel hydrazide column. After loading, the solid support was incubated with the serum for 30 mins at room temperature with end to end shaking at 9 rpm. Post this, the incubation column was washed with PBS followed by elution of the bound anti-idiotypic antibody using 20mM ortho-phosphoric acid. The eluted anti-idiotypic antibodies were neutralized using 0.2M disodium hydrogen phosphate. Further, the regenerate from the column was collected using 100mM ortho-phosphoric acid. Collected elute and regenerate were pooled, concentrated and buffer exchanged with PBS using 15 ml centrifugal filter devices (30kDa) to a total volume of 8ml and used as load in next stage of purification. The concentrate obtained from the above step was loaded on to a human IgG coupled affigel hydrazide column and was incubated for 30 mins at room temperature with end to end shaking at 9rpm. After incubation, flow through was collected in a separate tube. Elution of bound non-specific anti-idiotypic

antibody is followed using 20mM ortho-phosphoric acid and column was regenerated using 100mM ortho-phosphoric acid. The flow through collected from the above step was added to the human IgG coupled NHS activated sepharose and was incubated for 30 mins at room temperature with end to end shaking at 9 rpm. After incubation, flow through was collected from column and concentrated using 2ml centrifugal filters (30 KDa) (Format-1).

Further, two different series of purification were employed using the same three column in different combinations. All columns were pre-equilibrated with PBS. One such series of purification include loading of sera onto a human IgG coupled NHS activated sepharose column and flow through from the column was collected and loaded on to rituximab coupled affigel hydrazide column and was incubated for 30 mins and the bound anti-idiotypic antibodies were eluted using 20mM ortho-phosphoric acid. This was neutralized using 0.2M disodium hydrogen phosphate. Further, the column was regenerated using 100mM ortho-phosphoric acid. Collected elute and regenerate were pooled and concentrated and buffer exchanged with PBS using 15 ml centrifugal filter devices (30kDa) to a total volume of 8ml. The obtained concentrate from the above step was loaded on to a human IgG coupled affigel hydrazide and was incubated for 30 mins. After incubation, the flow through collected from the column was concentrated using 2ml centrifugal filters (30 KDa) to obtain a purified anti-idiotypic antibody (Format-2).

One another series of purification was also evaluated, wherein the sequence of columns used for purification of anti-idiotypic antibody was different and this series include human IgG coupled affigel hydrazide followed by rituximab coupled affigel hydrazide column followed by human IgG coupled NHS activated sepharose (Format-3).

Anti-idiotypic antibodies were obtained from the three different series of purification was measured at 280 nm and diluted to different concentrations prior to the subjecting of obtained sample for cross reactivity.

Cross reactivity of antibody:

To assess the cross reactivity of the obtained anti-idiotypic antibodies with human IgG, various wells of 96 well micro titer plate was coated with 100 µl of 1µg/ml of rituximab and human IgG on separate wells and incubated for 1 hr at room temperature with end to end shaking at 300rpm. After coating, wells were washed with PBS buffer containing Tween® (PBST) for three times to remove uncoated rituximab and human IgG followed by blocking with 3% BSA. Post blocking, wells were washed with PBS to remove unbound BSA followed by addition of 100 µl of samples obtained from the three different series of purifications (Format 1, Format 2, Format 3). The samples contain various concentrations of anti-idiotypic antibodies raised against rituximab and was incubated for 1 hr at room temperature with end to end shaking at 300rpm. After incubation, wells were washed several times with PBST to remove unbound anti-idiotypic antibody. 100 µl of anti-rabbit antibody Horse Radish Peroxidase conjugated (which was diluted in 1: 80000) was added to the plates and incubated for 1 hr at room temperature with end to end shaking at 300rpm. The wells were washed with PBST buffer for three times and 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added and incubated at room temperature for 10 minutes. 100 µl of stop solution was added to stop the reaction and absorbance at 450 nm was measured using a micro plate reader.

Specificity (inverse of cross reactivity) of the samples was calculated by following formula and was measured in terms of S/N ratio and was represented in table 1.

Specificity = Mean absorbance of samples incubated with rituximab (S)/ Mean absorbance of samples incubated with rituximab (N)

Table 1: Indicates specificity of the various concentration of anti-idiotypic antibodies in terms of S/N ratio

Concentration of Sample (ng/ml)	S/N ratio		
	Format-1	Format-2	Format-3
1000	11	3	10
500	23	4	18
250	39	6	23

From the above results, it is evident that higher S/N ratios are obtained with the anti-idiotypic antibodies obtained from format-1 followed by format-3 and format-2. Higher S/N ratios are obtained in format-1 indicates the higher specificity of the purified anti-idiotypic antibodies against rituximab which in turn indicates the lesser cross-reactivity.

CLAIMS

We Claim:

1. A method of purifying anti-idiotypic antibodies in sample using affinity chromatography, wherein the said method comprises sequential purification steps performed in columns comprising targeted antibody coupled affigel hydrazide column, followed by human IgG coupled affigel hydrazide column and followed by human IgG coupled NHS activated sepharose to obtain highly specific anti-idiotypic antibodies.

2. A method of purifying highly specific anti-idiotypic antibodies raised against a targeted antibody in sample, comprising;

a) loading the serum containing anti-idiotypic antibody on a targeted antibody coupled affigel matrix, followed by eluting the said bound anti-idiotypic antibodies with an acidic buffer followed by neutralization;

b) loading the neutralized elute obtained in step a) onto a human IgG-coupled affigel matrix followed by collecting the flow through containing anti-idiotypic antibodies and,

c) loading the collected flow through obtained in step b) onto a human IgG coupled NHS activated sepharose matrix followed by collecting the flow through containing purified anti-idiotypic antibody, wherein the purified anti-idiotypic antibody, obtained by the method of the present invention is highly specific, and contains negligible cross reactivity with other antibodies.

3. The method according to claim 1 or 2, the target antibody is selected from any of the groups like chimeric, humanized and human therapeutic antibodies.

4. The sample according to claim 1 or 2, is serum comprising the raised anti-idiotypic antibodies.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
C07K1/22,C07K1/18 Version=2015.01

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 practicable, search terms used)

IPO-INTERNAL, PATSEER

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008061684 A1 (HOFFMANN LA ROCHE) 29.05.2008 (29 May, 2008)	2-4
Y	page 18 lines 25-31, page 19, lines 1-16	1-4
Y	EP 0525570 A2 (BAYER CORPORATION) 03.02.1993 (03 February, 1993) paragraph [0018]	1-4
A	SOUZA A R et al: "Anti-Idiotypic Antibodies in Patients with Different Clinical Forms of Paracoccidioidomycosis", Clinical And Diagnostic Laboratory Immunology, Vol. 7, No. 2, Mar. 2000, pages: 175-181. whole document	1-4

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

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Information on patent family members

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WO 2008061684 A1	29-05-2008	AU 2007323364 B2	07-03-2013
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