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(21) International Application Number: PCT/US91/00942 (22) International Filing Date: 12 February 1991 (12.02.91) (30) Priority data: 482,215 20 February 1990 (20.02.90) US (71) Applicant: COULTER CORPORATION [US/US]; 600 West 20th Street, Hialeah, FL 33010 (US). (72) Inventor: RAVINDER, K., Gupta ; 9430 N.W. 19th Street, Pembroke Pines, FL 33024 (US). (74) Agents: CASS, Myron, C. et al.; Silverman, Cass & Singer, Ltd., 105 West Adams Street, 27th Floor, Chicago, IL 60603 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report</i> <i>With amended claims.</i>
(54) Title: IMPROVED ANTIBODY-ENZYME DIRECT CONJUGATES AND METHOD OF MAKING SAME (57) Abstract A direct antibody-enzyme conjugate which enzyme to antibody ratio is approximately 3. The method of producing the direct antibody-enzyme conjugate comprises adding sulfhydryl groups to the antibody and maleimide groups to an enzyme and then conjugating the so-modified antibody and enzyme.		

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IMPROVED ANTIBODY-ENZYME DIRECT CONJUGATES AND METHOD OF MAKING SAME

TECHNICAL FIELD

This invention relates generally to enzyme immunoassays and immunochemistry involving antibody-enzyme conjugates, and more particularly, relates to an improved antibody-enzyme direct conjugate for use in such assays which provides an enhanced enzyme-to-antibody ratio for a desirable high degree of sensitivity, and to the method for making the conjugate.

BACKGROUND ART

Methods for detecting the presence and/or quantity of an immunoreactant, such as an antibody or antigen, present in a human physiological fluid or tissue test sample often employ labelled reagents in a specific complexing process for the detection of these immunoreactants. Commonly used labels include radioactive, enzymatic, fluorescent and chemiluminescent labels. The label chosen for a particular detection method often depends upon the balancing of the advantages and disadvantages of the labels under consideration. Factors such as label size, label stability, available instrumentation, practicability and sensitivity influence the choice of label. See R.M. Dauphinais, Solving and preventing problems in ligand assay, in N.R. Rose et al., Manual of Clinical Laboratory Immunology, Third Edition, Ch. 16, pp. 88-98 (1986).

The avidin-biotin labelling technique has been shown to provide a high level of sensitivity. E.A. Bayer et al., The use of avidin-biotin complex as a tool in molecular biology. Methods Biochem. Anal. 26:1-43 (1980). Avidin is a basic glycoprotein derived from egg albumin which has a molecular weight of 68,000 daltons. It has a high affinity for the vitamin biotin which has a molecular weight of approximately 244 daltons. Each avidin molecule has four binding sites for biotin. The avidin-biotin sys-

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tem employs an immunoreactant which is usually an antibody linked to biotin and a label which is usually an enzyme linked to avidin. The strong interaction between avidin and biotin is used to link the biotinylated antibody to the enzyme-labelled avidin, thus serving as a means of amplifying the sensitivity of the immunoassay in which it is so used. The avidin-biotin labelling system thus has been employed to enhance sensitivity over other labelling systems, including the direct enzyme conjugate labelling system. U.S. Patent No. 4,228,237 describes a method to determine the presence of a ligand, such as, a labelled antibody, in a liquid medium which utilizes enzyme-labelled avidin and a biotin labelled reagent. Also, U.S. Patent No. 4,684,609 teaches a biotin-avidin label and its use for labelling tissue sections.

Although the avidin-biotin labelling system can provide an enhanced signal over previous labelling systems, problems are encountered with its use. Since the biotin and avidin usually are coupled with a ligand and a label, an additional incubation step and additional washing steps are required to be performed. Furthermore, the hydrophobic nature of the biotin molecule gives rise to higher background "noise" and false positive reactions in the performance of an assay method which utilizes avidin and biotin. Background noise is herein defined as the binding of the labelled species or fragments thereof to reagents in the assay system which causes non-specific precipitation of interference that is unrelated to the specific protein-bound fraction.

Direct antibody-enzyme conjugates are well known in the immunoassay art and have been described in the literature. See, for example, E. Ishikawa et al., J. Immunochimistry 4(3):209-327 (1983). The degree of sensitivity obtained with these direct antibody-enzyme conjugates, however, often is much lower than the degree of sensitivity obtained with the avidin-biotin system. This lower degree of sensitivity is due to the low enzyme-to-

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antibody ratio obtained for these conjugates in these preparations. In fact, the majority of these direct antibody-enzyme conjugate preparations give enzyme-to-antibody ratios of 0.3 to 1.0 (E. Ishikawa, *ibid*, page 221). Higher enzyme-to-antibody ratios usually are not desirable, if obtainable, because of the potential loss in antibody activity and higher background "noise".

It would be advantageous to provide a labelling system wherein direct antibody-enzyme conjugates could achieve high enzyme-to-antibody ratios and therefore provide a higher degree of sensitivity approaching or equal to the avidin-biotin labelling system. Such an improved direct antibody-enzyme conjugate would not require the additional incubation step and washing steps as the biotin-avidin labelling system requires. Also, such a system would avoid the problems of using a hydrophobic molecule such as biotin, and therefore, lessen the chance of higher background "noise" and false positive reactions.

The invention herein provides for such a improved direct antibody-enzyme conjugate and a labelling system for direct antibody-enzyme conjugates which achieves a high enzyme-to-antibody ratio of approximately three (3) without any significant loss of antibody activity or higher back ground "noise". This conjugate was prepared by using long chains between the antibody and enzyme molecules. In the procedure, sulfhydryl groups are introduced to the antibody. Additionally, maleimide groups are introduced into an enzyme, for example, Horseradish Peroxidase. This modified antibody and modified enzyme are combined and allowed to react. A mean enzyme-to-antibody ratio of approximately three (3) is achieved. A higher level of sensitivity is provided by the improved method without any increase in background "noise". Thus, one incubation and several washing steps required by the biotin-avidin labelling system are eliminated by the labelling system of this invention. It also eliminates the false positive reactions obtained by the use of hydro-

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phobic biotin molecule in the biotin-avidin enzyme indirect method described hereinabove.

DISCLOSURE OF THE INVENTION

A direct antibody-enzyme conjugate is provided which achieves a high enzyme-to-antibody ratio of approximately three (3) and which, therefore, provides a higher degree of sensitivity than heretofore seen with direct antibody-enzyme conjugates.

The conjugate of the invention is prepared by a novel procedure wherein sulfhydryl groups are introduced to the antibody and maleimide groups are introduced to the enzyme. The thus-modified antibody and enzyme are combined in a suitable ratio to provide the direct antibody-enzyme conjugate.

BEST MODE FOR CARRYING OUT THE INVENTION

The method of the invention is practiced by performing a 3-step procedure. In STEP A, the antibody is modified by introducing sulfhydryl groups to the antibody. In STEP B, maleimide groups are introduced to the enzyme by using sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate. In STEP C, direct conjugation is performed by suitably adapting a method described by S. Yoshitoba et al., Eur. J. Biochem. 1-1:395-399 (1979).

A glossary of reagents employed in practicing the invention is as follows:

STEP A:

Antibody, 20 mg/ml in 100mM Phosphate,
150mM NaCl, 2mM EDTA, pH 7.4
15 Molar excess of 2-iminothiolane hydrochloride
Sephadex® G-50 Column

STEP B:

Horseradish Peroxidase (Sigma Chemical Co.)
DEAE Sepharose CL-6B column (Pharmacia)
2.5mM Potassium Phosphate, pH 8.0
Sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate
at 15 M excess

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100mM Potassium Phosphate, 100mM NaCl, pH 7.4

STEP C:

Modified antibody from STEP A

Modified peroxidase from STEP B

Cysteine

Iodoacetamide, pH 8.7

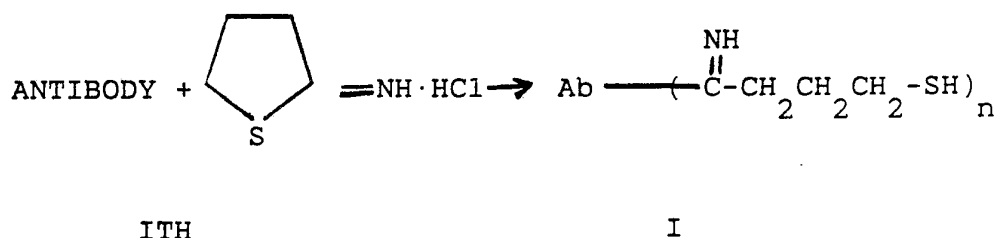
Amicon YM-30 membrane (W.R. Grace & Co.)

TSK-250 FPLC column

CONJUGATION PROCEDURE:

STEP A:

A solution of a selected antibody (Ab) at 20 mg/ml in 100mM phosphate, 150mM NaCl, 2mM EDTA, pH 7.4, is treated with a 15 molar excess of 2-iminothiolane hydrochloride (ITH) for one hour at 22°C. Then the reaction mixture is desalted over a Sephadex® G-50 column. The product protein peak (I) is collected and its concentration is determined from the A280 value. The conjugating mechanism is represented diagrammatically as follows:



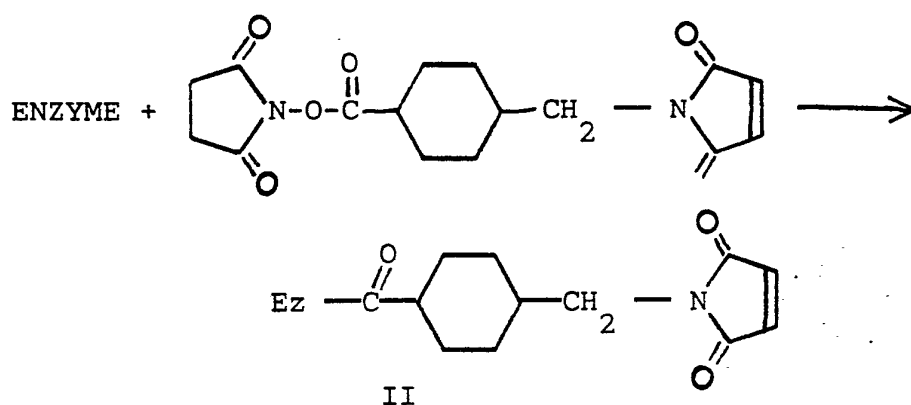
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STEP B:

An enzyme (Ez) such as Horseradish Peroxidase (Sigma Chemical Co.) is purified over a DEAE Sepharose CL-6B column (Pharmacia) in 2.5mM Potassium Phosphate buffer, pH 8.0, and the pass through peak is collected. Purified horseradish peroxidase (at 13 mg/ml) is reacted with sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate, SMCC, (Pierce Chemical Co.) at 15 molar excess

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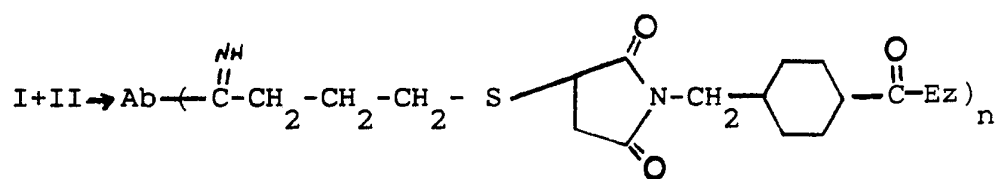
in 100 mM Potassium Phosphate buffer, 100mM NaCl pH 7.4 for one hour at between 21° and 25° C. The reaction mixture next is desalted over a Sephadex ®G-50 column and the product protein (II) peak is collected. The concentration of the protein peak is calculated for absorbence at 403nm. The reaction mechanism is represented diagrammatically as follows:



STEP C:

Modified antibody and modified peroxidase obtained by performing STEPS A and B as described herein next are combined with stirring to provide a four molar excess of peroxidase over antibody. This excess of modified peroxidase to modified antibody was chosen to produce a product having an enzyme-to-antibody ratio of approximately three (3). The reaction then is allowed to take place at 22°C over two hours. (This conjugation procedure can be varied with respect to the time and temperature and still produce a conjugate with an enzyme-to-antibody ratio of approximately three (3).) The reaction is quenched by addition of cysteine to a 5mM concentration. After 15 minutes the reaction is further quenched by the addition of excess iodoacetamide at pH 8.7 for 30 minutes. The reaction mixture is concentrated over a YM-30 membrane and then it is chromatographed over a TSK-250 FPLC column. The protein peak is collected into various fractions and analyzed on a spectrophotometer. The conjugating mechanism is represented diagrammatically as follows:

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The enzyme used in this direct antibody-enzyme conjugate can vary. Examples of suitable enzymes for use in practicing the invention include horseradish peroxidase, alkaline phosphatase, galactose, oxidase and lactoperoxidase. Various antibodies can be employed which include polyclonal and monoclonal antibodies. Antibody fragments also can be employed. The human anti-HIV antibody was chosen for the example given below. In addition to 2-iminothiolane hydrochloride, other sulfhydryl producing reagents useful in practicing this invention include N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and N-succinimidyl-S-acetyl-thioacetate (SATA). Additional reagents capable of introducing maleimide groups include succinimidyl 4-(p-maleimidylphenyl) butyrate, m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester, succinimidyl maleimidylacetate, and succinimidyl 6-maleimidylhexanoate.

A preferred embodiment is described by the following example:

EXAMPLE

A solution of human anti-HIV antibody was prepared as described in STEP A. Horseradish peroxidase was purified and reacted as described in STEP B. The modified human anti-HIV antibody and modified horseradish peroxidase were combined as described in STEP C. The first peak from the column gave the conjugate with a enzyme-antibody ratio value of 2.93.

The preferred embodiment can be varied by substituting the reagents specifically identified herein. The preferred ratio value of approximately 3 is to be noted.

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CLAIMS

1. A method for producing a direct antibody-enzyme conjugate, which method comprises:
 - a. modifying the antibody by introducing sulfhydryl groups to the antibody, desalting the antibody and collecting its protein peak;
 - b. modifying the enzyme by purifying the enzyme, introducing maleimide groups to the enzyme, desalting the enzyme and collecting its protein peak;
 - c. combining and reacting the modified antibody of (a) with the modified enzyme of (b) for a time and under conditions sufficient for complexes to be formed between the modified antibody and the modified enzyme; and
 - d. concentrating and collecting the protein peak of the antibody-enzyme conjugate of (c).
2. The method according to claim 1 wherein the antibody is a polyclonal antibody.
3. The method according to claim 1 wherein the antibody is a monoclonal antibody.
4. The method according to claim 1 wherein the antibody comprises an antibody fragment.
5. The method according to claim 1 wherein the enzyme is horseradish peroxidase.
6. The method of claim 1 wherein the reactions occur at between approximately 21-25° C.
7. The method of claim 1 wherein the ratio of enzyme to antibody of the protein peak of said antibody-enzyme conjugate collected in step (d) is approximately 3.
8. A direct antibody-enzyme conjugate, which conjugate comprises an antibody with sulfhydryl groups attached to it and an enzyme with maleimide groups attached to it, wherein the ratio of enzyme to antibody of the protein peak of said direct antibody-enzyme conjugate is approximately 3.
9. The conjugate of claim 7 wherein said antibody is a

polyclonal antibody.

10. The conjugate of claim 7 wherein said antibody is a monoclonal antibody.

11. The conjugate of claim 7 wherein said antibody is an antibody fragment.

12. The conjugate of claim 7 wherein said enzyme is horseradish peroxidase.

13. A direct antibody-enzyme conjugate which comprises combining an antibody having sulfhydryl groups attached to it with an enzyme having maleimide groups attached to it, for a time and under conditions sufficient for a reaction to occur between said antibody and said enzyme, and wherein the ratio of enzyme to antibody of said antibody-enzyme conjugate is approximately 3.

14. The conjugate of claim 13 wherein said antibody is a polyclonal antibody.

15. The conjugate of claim 13 wherein said antibody is a monoclonal antibody.

16. The conjugate of claim 13 wherein said antibody is an antibody fragment.

17. The conjugate of claim 13 wherein said enzyme is horseradish peroxidase.

18. The conjugate of claim 13 wherein said reaction occurs at between approximately 21-25°C.

AMENDED CLAIMS

[received by the International Bureau
on 9 August 1991 (09.08.91);

original claims 1, 8 and 13 amended; other claims unchanged (2 pages)]

1. A method for producing an antibody-enzyme conjugate, which method comprises:
 - a. modifying the antibody by introducing sulfhydryl groups to the antibody, desalting the antibody and collecting the modified, desalted antibody containing said sulfhydryl groups;
 - b. modifying the enzyme by purifying the enzyme, introducing maleimide groups to the enzyme, desalting the enzyme and collecting the modified, desalted enzyme containing said maleimide groups;
 - c. combining and reacting the modified antibody of (a) with the modified enzyme of (b) for a time and under conditions sufficient for complexes having an enzyme-to-antibody ratio of approximately 3 to be formed between the modified antibody and the modified enzyme; and
 - d. concentrating and collecting the protein peak of the antibody-enzyme conjugate of (c).
2. The method according to claim 1 wherein the antibody is a polyclonal antibody.
3. The method according to claim 1 wherein the antibody is a monoclonal antibody.
4. The method according to claim 1 wherein the antibody comprises an antibody fragment.
5. The method according to claim 1 wherein the enzyme is horseradish peroxidase.
6. The method of claim 1 wherein the reactions occur at between approximately 21-25° C.
7. An antibody-enzyme conjugate, which conjugate comprises an antibody with sulfhydryl groups attached to it and an enzyme with maleimide groups attached to it, wherein the ratio of enzyme to antibody in said direct antibody-enzyme conjugate is approximately 3.
8. The conjugate of claim 7 wherein said antibody is a

polyclonal antibody.

9. The conjugate of claim 7 wherein said antibody is a monoclonal antibody.

10. The conjugate of claim 7 wherein said antibody is an antibody fragment.

11. The conjugate of claim 7 wherein said enzyme is horseradish peroxidase.

12. A antibody-enzyme conjugate produced by the step of combining an antibody having sulfhydryl groups attached to it with an enzyme having maleimide groups attached to it, for a time and under conditions sufficient for a reaction to occur between said antibody and said enzyme, and wherein the ratio of enzyme to antibody of said antibody-enzyme conjugate is approximately 3.

13. The conjugate of claim 12 wherein said antibody is a polyclonal antibody.

14. The conjugate of claim 12 wherein said antibody is a monoclonal antibody.

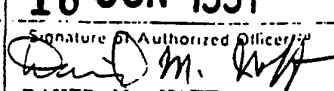
15. The conjugate of claim 12 wherein said antibody is an antibody fragment.

16. The conjugate of claim 12 wherein said enzyme is horseradish peroxidase.

17. The conjugate of claim 12 wherein said reaction occurs at approximately 21-25°C.

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US91/00942**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): G01N 33/535; C12N 11/00, 9/96; C07K 3/00 U.S. CL.: 435/7.9, 174, 188; 436/512, 548; 530/402		
II. FIELDS SEARCHED		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
U.S.	435/7.9, 174, 188; 436/512, 519, 541, 547, 548; 530/390, 402, 404, 806	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT 14		
Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
X Y	Journal of Applied Biochemistry, vol. 4, issued 1982, Imagawa et al., "Characteristics and Evaluation of Antibody-Horseradish Peroxidase Conjugates Prepared by Using a Maleimide Compound, Glutaraldehyde, and Periodate. see entire document, pp. 41-57.	1,2,4,5,7-9,11-14,16,17 3,6,10,15,18
Y,E	US,A, 5,002,883 (Bieniarz et al.) 26 March 1991, see entire document.	1-18
Y	US,A, 4,894,229 (Polson et al.) 16 January 1990, see entire document.	1-18
A	US,A, 4,228,237 (Hevey et al.) 14 October 1980, see entire document.	
A	US,A, 4,684,609 (Hsu) 04 August 1987, see entire document.	
* Special categories of cited documents: 15 "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed		
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IV. CERTIFICATION		
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08 MAY 1991		18 JUN 1991
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