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(54) Title: STABILIZED ENZYME SUBSTRATE SOLUTIONS

(57) Abstract

A solution comprising a chromogen or substrate for use in an enzyme immunoassay, enzyme linked immunosorbent assay, immunoenzymometric assay or immunoperoxidase assays is stabilized to substantially reduce substrate drift. This is achieved by the addition of a chelating agent in an amount effective to substantially prevent nonenzymatic oxidation of the substrate or chromogen.

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STABILIZED ENZYME SUBSTRATE SOLUTIONS

DESCRIPTION OF THE INVENTION:

This invention relates to an improved enzyme immunoassay (EIA), enzyme linked immunosorbent assay (ELISA), immunoenzymometric assay or immunoperoxidase assay and pertains most specifically to a substrate solution for determining enzyme activity which contains ethylenediaminetetraacetic acid resulting in increased stability of the substrate solution and decreased substrate drift.

Enzyme immunoassays, enzyme linked immunosorbent assays and immunoenzymometric assays involve the use of an enzyme, such as a peroxidase, as a label for the unknown in an assay procedure, and measurement of the enzyme activity as an indication of the amount of the unknown in the sample. Immunoperoxidase assays apply the same

principles to microscopic examination of cultures or tissues. Such assays typically require the addition of the substrate for the enzyme after the immunoassay in order to determine the amount of "bound" enzyme. Peroxidases, such as horseradish peroxidase, require two substrates -- a peroxide and a chromogen which affords a colored product upon oxidation. Typically, stock solutions of the peroxide and the chromogen are combined in the appropriate buffer to form a "working" substrate solution.

With the usual chromogen, o-phenylenediamine, the working solution is quickly oxidized nonenzymatically resulting in an increase in color development even in the absence of peroxidase. Thus, the working solution must be used within 1/2 hour of preparation.

A better chromogen, 3,3',5,5'-tetramethylbenzidine (TMB) has been developed. The working solution with TMB and peroxide also will be oxidized nonenzymatically, although more slowly. Thus the solution may be prepared one to two hours in advance of use. However, it has been noted that occasionally the working solution becomes colored very quickly, often within seconds. In such cases, the solution is not usable. This change in intensity of background color termed "substrate drift" greatly limits the utility of such substrate solution in enzyme immunoassay or enzyme linked immunosorbent assays.

I have now found that such nonenzymatic oxidation and, hence, color development may be prevented by the addition of a chelating agent to the stock solutions from which the working solution is made. Experiments have indicated that such nonenzymatic oxidation, may be due to contamination by heavy metals. The addition of ferric ions to the working solution cause immediate generation of blue color. The addition of chelating agent prevents the unidentified heavy metals from catalizing oxidation increasing stability of the substrate solution and decreasing substrate drift.

The enzyme-substrate system in which the present working solution is useful is any such system in which concentration or presence of the unknown is detected by enzymatic oxidation of the substrate. For example, the present invention can be used to determine peroxidase activity of any enzyme which catalyzes the reaction of the chromogen with peroxide to form a colored compound. Although peroxidases, such as horseradish peroxidase, are among the more widely used of such enzymes, other peroxidases may also be used.

"Chelating agent" as used in this invention means any compound in which a dicationic, tricationic or tetracationic metal is bound to two or more atoms or complexes with ligands containing more than one point of attachment. Chelating agents which can be used in the present invention include any chelating agent which binds heavy metals preventing nonenzymatic oxidation of the substrate solution without preventing enzymatic oxidation or the formation or detection of color. Preferred chelating agents are nontoxic and do not present a biohazard. These include, but are not limited to: ethylenediaminetetraacetic acid (EDTA) and other tetraacids, diethylenetriaminepentaacetic acid (DTPA) and other pentaacids, iminodiacetic acid and other derivatives, nitrilotriacetic acid and derivatives, succinic acid and other diacids, citric acid and other hydroxyacids and acetylacetone and other dicarbonyl compounds. Preferred chelating agents are EDTA and its derivatives, DTPA and its derivatives, iminodiacetic acid and its derivatives, and nitrilotriacetic acid and its derivatives. The most preferred is EDTA due to its solubility in the buffer of the stock and working solutions and its ability strongly to chelate a variety of metal ions at the pH of the working solution. Chelating agents which are not useful in the present invention are those which actually increase the rate of non-enzymatic oxidation of the chromogen or substrate, such as dipicolinic acid. These chelating agents will be obvious to one skilled in the art without undue experimentation.

The concentration of chelating agent in the working solutions will be dependent upon effective elimination of drift and minimization of interference with the enzymatic oxidation of the substrate. In general, it is desirable to minimize the concentration of chelating agent. Preferred concentrations are in the range of from about 0.01mM to about 100mM. The most preferred concentrations are in the range of from about 0.05mM to about 10mM.

Substrates and chromogens with which the present invention is useful include those which are used in the detection of peroxidases. Chromogens which are useful include those which are well known in the art such as o-phenylenediamine, 2,2'-azinodi (3-ethyl) benzthiazoline-6-sulphonic acid (ABTS), dianisidine, dicarboxidine, TMB, diaminobenzidine. Preferred chromogens include any 3,3',5,5'-tetraalkylbenzidine in which the alkyl groups each contain from 1 to 5 carbon atoms; particularly useful are 3,3',5,5'-tetramethylbenzidine and 3,3',5,5'-tetraethylbenzidine. Acid salts such as the hydrochlorides also are useful. The amount of chromogen present in the substrate solution can vary over a considerable range, depending upon the identity and concentration of the peroxidase enzyme whose activity is to be measured; in general, the concentration of chromogen can vary from about 0.1 to about 10 mM, preferably from about 1 to about 3mM. The amount of peroxide present also may vary, depending upon the amount of chromogen present, ranging from about 1 to about 20mM, but preferably it is from about 1 to about 6mM. Any of the usual peroxides such as hydrogen peroxide, urea peroxide, or the like can be employed in the substrate solution. It is also desirable to include a buffer in the solution, such as an acetate buffer, which maintains the pH from about 4 to about 7.

The working solution is prepared by mixing a first stock solution containing a chromogen and a chelating agent with a second stock solution comprising an aqueous buffer solution containing the

substrate and a chelating agent. The chelating agents of the two solutions may be the same or may differ. The chromogen solution can also comprise stabilizing or solubilizing agents. In the preferred peroxidase system, the working solution is prepared by mixing a first stock solution comprising an aqueous solution containing 5 to 50% N-methyl pyrrolidone by volume, 0.5 to 1mM EDTA and 1 to 10 mM 3,3',5,5'-tetraalkylbenzidine or an acid salt thereof with a second stock solution comprising an aqueous buffer solution containing 0.5 to 1mM EDTA and 4 to 40 mM peroxide, and water if necessary to achieve the desired concentration.

Stock solutions can be supplied in the form of a kit which contains in addition a supply of conventional stopping agent solution, antibody- or antigen-coated containers, and antigen or antibody standards, calibrators or controls.

The determination of enzyme activity is carried out in the usual manner by incubating the substrate solution with the specimen containing the enzyme to develop a visible color. For quantitative determinations the reaction with the substrate solution is stopped after an established time by adding a conventional stopping agent such as, for example, an aqueous solution of water-soluble flouride, such as sodium fluoride, at pH from about 3 to about 6; or acids such as sulfuric acid, hydrochloric acid, etc. A soluble fluoride is preferred because it provides a stable blue color.

The present invention can be employed with any of the usual enzyme immunoassay procedures, either homogeneous or heterogeneous assays, and either single- or double-antibody assays, and including enzyme linked immunosorbent assays, immunoenzymometric assays and immunoperoxidase assays.

The following examples are intended to illustrate more fully the nature of the present invention without acting as a limitation upon its scope.

EXAMPLE I

Aqueous substrate solutions were prepared with the following compositions:

A. 7.5 mM 3,3',5,5'-tetramethylbenzidine, 40% by volume N-methyl pyrrolidone.

B. 5 mM hydrogen peroxide, 100 mM acetate.

C. 7.5 mM 3,3',5,5'-tetramethylbenzidine, 40% by volume N-methyl pyrrolidone, 10^{-4}M EDTA.

D. 5mM hydrogen peroxide, 100 mM sodium acetate, 10^{-4}M EDTA.

The solutions were stored in glass at 4°C. Working substrate solutions with and without EDTA were prepared by combining one part of the respective TMB solution with 4 parts of the respective peroxide/acetate stock solution. The working solutions were stored in clear glass bottles at room temperature or at 4° to 6°C. At various intervals 200 µL (microliters) of the solution were acidified with 50 µl 1.0N H_2SO_4 and the absorbance at 450 nm was measured in a spectrophotometer using water as a blank standard. The results are recorded in Table 1.

Table 1

STABILITY OF WORKING SOLUTION

AT ROOM TEMPERATURE

Time After Preparation	Absorption at 450 nm Without EDTA	10^{-4}M EDTA
0	0.013	0.010
17 hr	0.625	0.007
5 d	1.820	0.053
7 d	0.551	0.097
AT 4 to 6°C		
0	0.010	0.010
2 hr	0.073	0.012
4 d	1.366	0.010
6 d	1.965	0.020

As can be seen from the data, the working substrate solution which contained EDTA was very stable for several days at 4 to 6°C and at room temperature. Without EDTA the nonenzymatic oxidation was so rapid that after a few hours the solution was no longer usable. The drop in absorbance at 7 days is probably due to the decomposition of the colored product occurring at a faster rate than it is formed.

EXAMPLE II

The effect of EDTA on enzyme activity was determined by running a horseradish peroxidase assay and varying the amount of horseradish peroxidase (1ng/ml). 400 µl of the working solution of Example I, with and without EDTA, were added. The reaction was stopped after 10 minutes with 1 ml of 1 M H_2SO_4 and the absorbances at 450 nm were determined as before.

TABLE 2

ENZYME ACTIVITY

Volume of HRP(µl)	Absorption at 450 nm No EDTA	10^{-4}M EDTA
0	0.015	0.004
10	0.117	0.156
20	0.243	0.288
40	0.503	0.600
60	0.826	0.875

Results show that EDTA had very little effect on the enzyme activity.

EXAMPLE III

Enzyme immunoassays were performed using the EDTA stabilized TMB/peroxide system for quantitation of carcinoembryonic antigen (CEA). CEA standards were prepared containing 0, 2, 5, 10 and 25 ngm/ml of CEA in serum respectively. 100 µl of each standard were added along with 100 µl of 50 nM acetate pH 5.3 buffer to separate IgG (anti-CEA) coated microtiter wells. The assays were incubated for 2.0 hours at 37°C. Each well was washed three times with water. A conjugate was formed by covalently linking A'-CEA IgG to enzyme horseradish peroxidase (HRP) diluted in buffer with protein stabilizer. 200 µl of the conjugate was added to each well. The assays were further incubated for 1.0 hour at 37°C. The wells were washed with water as before and 200 µl of EDTA stabilized working solution prepared as in Example I was added to each well. The assays were incubated for 30 minutes at 20 to 23°C. The reaction was stopped by the addition of 50 µl per well of 0.1% NaF pH 3.5. The wells were then read in a microtiter spectrometer at 650 nm (See Table 3).

TABLE 3

QUANTITATION OF CEA BY EIA

CEA Concentration Ngm/ml	Absorbance (650 nm)
0	0.037
2	0.142
5	0.311
10	0.625
25	1.58

EXAMPLE IV

Working solutions were prepared as in Example I varying the chelating agent. The solutions with and without 10^{-4}M chelating agent were stored in clear glass bottles at room temperature. At various times 200 µl of the solution were used to determine absorbance at 650 nm. Stop solution (1.0 N H_2SO_4) was not used. The results are shown in Table 4.

TABLE 4

STABILITY OF WORKING SOLUTION AT ROOM TEMPERATURE

Chelating Agent (10^{-4} Molar)	Absorption at 650 nm Time after Preparation				
	0	2 hr.	6 hr.	1 d	3 d
NONE	0.018	0.063	0.145	0.158	0.845
EDTA	0.015	0.015	0.017	0.015	0.039
DTPA	0.015	0.019	0.021	0.015	0.042
Iminodiacetic Acid	0.014	0.020	0.024	0.043	0.102
Dipicolinic Acid	0.031	0.124	0.219	0.490	0.879
Citric Acid	0.020	0.020	0.024	0.050	0.053

The data shows that all of the chelating agents except dipicolinic acid prevented rapid non-enzymatic oxidation of the solution. Dipicolinic acid, on the other hand, increased non-enzymatic oxidation relative to the control and would not be suitable for use as a stabilizer.

EXAMPLE V

To test the effect of chelating agents on enzyme sensitivity, enzyme immunoassays for Hepatitis B surface antigen (HBsAg) were run using a TMB/Peroxide substrate system prepared with and without varying chelating agents. Controls were prepared using HBsAg diluted in buffer with 1% bovine serum albumin as a positive and human serum as a negative. 200 µl of control were added to anti-BHsAg coated microtiter wells. 200 µl of water was used for the blank. The wells were incubated for 2 hours at 37°C and then washed three times with water. 200 µl of a A'-BHsAg-HRP conjugate comprised of anti-HBsAg IgG covalently linked to horseradish peroxidase diluted in buffer with protein stabilizer was added to each well. The wells were incubated for one hour at 37°C and then washed as before. To each well was added 200 µl of working solution, with or without the chelating agent. The wells were incubated at 20-23°C for 30 minutes. The reaction was then stopped by the addition of 50 µl per well of 0.1% NaF pH 3.5. Absorbances were read in a microtiter spectrophotometer at 650 nm. The results are given in Table 5.

TABLE 5

HBsAg ENZYME IMMUNOASSAY

Chelating Agent Added to Substrate	Blank	HBsAg Controls Negative	Positive
None	0.042	0.013	1.057
EDTA	0.033	0.008	1.038
DTPA	0.030	0.008	1.035
Iminodiacetic Acid	0.032	0.028	1.022
Dipicolinic Acid	0.424	0.008	0.686
Citric Acid	0.033	0.011	1.026

Results suggest little effect on the assay sensitivity by most of the chelating agents. Dipicolinic acid appears to reduce assay sensitivity by about 50% and, therefore, is not acceptable for use in this invention.

WHAT IS CLAIMED IS:

1. A solution for use in an enzyme-immunoassay, enzyme linked immunosorbent assay, immunoenzymometric assay or immunoperoxidase assay, in which a chromogen and a substrate are reacted with an enzyme in an aqueous or mixed aqueous-organic buffer, which comprises a substrate or a chromogen and an effective amount of chelating agent to substantially prevent nonenzymatic oxidation of the substrate or chromogen.

2. The solution of Claim 1 wherein the substrate is peroxide and the enzyme is peroxidase.

3. The solution of Claim 2 wherein the chelating agent is selected from the group consisting of ethylenediaminetetraacetic acid (EDTA) and other tetraacids, diethylenetriaminepentaacetic acid (DTPA) and other pentaacids, iminodiacetic acid and other derivatives, nitrilotriacetic acid and derivatives, succinic acid and other diacids, citric acid and other hydroxyacids and acetylacetone and other dicarbonyl compounds.

4. The solution of Claim 2 wherein the chelating agent is selected from the group consisting of ethylenediaminetetraacetic acid, diethylenetriaminepentaacetic acid, iminodiacetic acid and citric acid.

5. The solution of Claim 4 wherein the chelating agent is ethylenediaminetetraacetic acid.

6. The solution of Claim 2 wherein the concentration of chelating agent is between from about 0.01mM to about 100mM.

7. The solution of Claim 6 wherein the concentration of chelating agent is between from about 0.05mM to about 10mM.

8. The solution of Claim 2 wherein the chromogen is selected from the group consisting of o-phenylenediamine, 2,2'-azinodi(3-ethyl)benzthiazoline-6-sulphonic acid (ABTS), dianisidine, dicarboxidine, diaminobenzidine, any 3,3', 5,5'-tetraalkylbenzidine in which the alkyl groups each contain from 1 to 5 carbon atoms, and the acid salts thereof.

9. The solution of Claim 8 wherein the chromogen is 3,3',5,5'-tetramethylbenzidine.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/00229

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.4 C12Q 1/00,G01N 33/53,C12Q 1/28,C12N 9/96;G01N 1/00
U.S. CL. 435/4,7,28,188,810; 436/825,826

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/4,7,28,188,810 436/825,826

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

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	US,A, 4,188,465 (Schneider et al) 12 Feb. 1980	1-9
X	US,A, 4,282,316 (Modrovich) 4 Aug. 1981	1-9
A	US,A, 4,378,429 (Modrovich) 29 Mar. 1983	1-9
X	US,A, 4,331,761 (Dawson et al) 25 May 1982	1-9
X	JP,A, 0034001 (NODA INST. SCI. RES) 10 Mar. 1980	1-9

* Special categories of cited documents: [15]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
16 April 1986	21 APR 1986
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/U.S.	Patricia Kate White PATRICIA KATE WHITE

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
X	JP,A, 0138389 (TOYOBO KK) 26 Aug. 1982	1-9
X	JP,A, 0162294 (TOYOBO KK) 26 Sept. 1983	1-9
X	DE,A, 3124590 (BOEHRINGER MANNHEIM GMBH) 27 Jan 1983	1-9
X	EP,A, 0043550 (BEHRINGWERKE AG) 13 Jan 1982	1-9