REAGENT COMPOSITION AND PROCESS FOR THE DETERMINATION OF GLUCOSE

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References Cited

FOREIGN PATENTS OR APPLICATIONS

283,281 7/1970 Austria

OTHER PUBLICATIONS


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ABSTRACT

Compositions comprising glucose oxidase, peroxidase, a chromogen, a buffer, an azide, and 2,2'-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid) provide remarkably stable test reagents for the enzymatic determination of glucose.

13 Claims, No Drawings
REAGENT COMPOSITION AND PROCESS FOR THE DETERMINATION OF GLUCOSE

The present invention is concerned with a reagent composition and process for the enzymatic determination of glucose by use of the enzymes glucose oxidase and peroxidase.

In carrying out the determination of glucose with the conventional use of glucose oxidase, hydrogen peroxide is formed according to the equation:

\[ \text{glucose} + \text{O}_2 + \text{H}_2\text{O}_2 \rightarrow \text{gluconic acid} + \text{H}_2\text{O} \]

This hydrogen peroxide oxidizes, according to this method, in the presence of peroxidase, a chromogen, for example, o-dianisidine, with a change of color. The oxidized chromogen is then determined and gives a measure of the glucose which has participated in the reaction.

In the case of test reagents, it is generally desired that these contain all the necessary reagents in a pre-mixed state in order, on the one hand, to reduce as much as possible the expenditure of labor in carrying out large numbers of routine investigations and, on the other hand, to prevent errors arising in the mixing of several components. For reasons of easier dispatch and storage, as well as of superior stability, these reagent mixtures should be in solid form but should be easily dissolved in water at the time of use and then remain stable in aqueous solution for as long as possible in order to avoid having to prepare new solutions continuously.

However, the previously known reagent mixtures for the determination of glucose by the glucose oxidase (GOD)/peroxidase (POD) method did not fulfill these requirements. In particular, it was found that stabilization with a preservation agent, such as an azide, which was normally well compatible with enzymes, was not possible because of the rapid decrease of the POD activity which occurred in the solution. Therefore, stabilization with an azide was not possible and the known unstabilized reagent mixtures could heretofore only be used for a short time after dissolving in water.

It has been found that this disadvantage can be overcome when a reagent of this type contains, as a stabilizer an azide together with 2,2'-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid).

Therefore, the present invention provides a reagent composition for the determination of glucose, which comprises glucose oxidase, peroxidase, a chromogen and a buffer, together with an azide and 2,2'-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid), hereinafter abbreviated as ABTS.

The use of ABTS as a chromogen for carrying out glucose determinations by the GOD/POD method has already been described in German Pat. specification No. 1,648,840. However, its use in combination with an azide is new and it was not to have been expected that a mixture of an azide and ABTS would exert a stabilizing action on the reagent, since neither of the two substances alone exhibits such a stabilizing action.

In the reagent according to one aspect of the present invention, ABTS can be used simultaneously as stabilizer and as the chromogen. In this case, it is expediently present in the reagent in an amount of 0.25 to 1.5 grams/liter, preferably of about 0.5 grams/liter, of aqueous solution or, in the case of a solid mixture intended for the preparation of one liter of solution, is present therein in an equivalent amount. If another chromogen is used, for example o-dianisidine, then a substantially smaller amount of ABTS suffices for the achievement of the desired stabilizing action in admixture with an azide, which amount is about 1/200th of the above-given amount, i.e., 1 to 10 mg./liter and preferably 2 to 5 mg./liter.

The azide, which is preferably an alkali metal azide, for example sodium azide, is preferably used in an amount of 0.05 to 0.2 grams/liter of reagent solution or of a corresponding amount in the case of a solid reagent mixture.

The enzymes GOD and POD are expediently present in the reagent according to the present invention in amounts such that the mutarotation of the glucose and not the enzymes determines the rate of the reaction. This requirement is fulfilled by GOD concentrations of 2.5 to 15 x 10^-3 U/liter (i.e., units per liter) and of POD concentrations of 30 to 1000 U/liter.

The buffer substance used should produce, in aqueous solution, a pH value of between 6.2 and 7.5 and preferably of between 6.5 and 7.2. All buffer substances can be used which do not inhibit the reaction, a phosphate buffer (pH 7.0) being preferably used. However, tris buffer is unsuitable.

Therefore, a preferred reagent according to the present invention for the determination of blood sugar comprises:

buffer substance, pH 6.2 to 7.5,
2.5 to 15 x 10^-3 U/liter GOD,
30 to 1000 U/liter POD,
0.05 to 0.2 grams/liter of an azide, and
0.25 to 1.5 grams/liter or 1 to 10 mg./liter ABTS
and optionally at least one additional chromogen, preferably in an amount of 50 to 70 mg., in the form of an aqueous solution or of a solid substance mixture.

An especially preferred reagent according to the present invention comprises:

0.1M phosphate buffer, pH 6.5 to 7.2,
9 to 12 x 10^-3 U/liter GOD,
150 to 500 U/liter POD,
0.1 grams/liter of an azide, and
0.5 grams/liters ABTS.

A further preferred reagent according to the present invention comprises:

0.1M phosphate buffer, pH 6.5 to 7.2,
9 to 12 x 10^-3 U/liter GOD,
150 to 500 U/liter POD,
0.1 grams/liter of an azide,
2 to 5 mg./liter ABTS and
50 to 70 mg./liter o-dianisidine.

As indicated above, the reagent according to the present invention can be in the form of an aqueous solution of the components. This aqueous solution has a good stability and can, depending upon the composition and especially upon the chromogen used, be stored ready for use for several weeks. In this lies a substantial improvement in comparison with the previously usual reagents in which, on the one hand, chromogen and enzymes had to be prepared in different solutions and, on the other hand, these solutions only had a limited period of use. Thus, it is now no longer necessary to prepare several reagent solutions, each of which can only be used for a short period of time: the solutions
3,721,607

preparing from the known reagents could not be used for longer than 1 day.

However, the reagents according to the present invention, whether in solid form or in the form of solutions, have several advantages, especially an outstanding stability. Their storage at refrigerator temperature is more than one year. Furthermore, solid mixtures according to the invention, e.g., in the form of powders or possibly of tablets, have the advantage of being very easily soluble in water.

A further surprising advantage of the reagent according to the present invention is that the amount of POD can be substantially reduced from the previously used amount of about 250 U/liter to about 30 U/liter. Nevertheless, POD does not become the Velocity-determining reaction component. This enables a substantial saving of POD.

The following Examples are given for the purpose of illustrating the present invention:

EXAMPLE 1

In a ball mill there were ground:
9.5 grams secondary sodium phosphate,
4.6 grams primary sodium phosphate monohydrate,
12 milligrams glucose oxidase,
40 milligrams peroxidase,
0.1 grams sodium azide and
0.5 grams ABTS
and subsequently well mixed together. The reagent mixture thus obtained was sufficient for the preparation of 1 liter of reagent solution. The stability of the solid reagent mixture in this form at 4°C. was more than one year.

For the determination of glucose, for example in blood, a reagent solution was prepared by dissolving 1.5 grams of the above solid powder mixture in 100 ml. water. This solution contained all reagents necessary for the determination of the glucose and, when stored at 4°C. can be used for at least 8 weeks.

5 ml. of the reagent solution were mixed with 0.2 ml. of the glucose-containing solution (obtained, for example, by the deproteinization of blood or serum with a known deproteinisation agent, such as perchloric acid or uranyl acetate). The mixture was left to stand for 25 minutes at ambient temperature and then the intensity of the colored material formed was measured. This was proportional to the glucose concentration present in the test solution. By means of a standard sample of known glucose concentration, which was determined at the same time, the desired glucose concentration of the test solution can easily be calculated.

EXAMPLE 2

Stability of the peroxidase

The reagent solution described in Example 1 was compared with a reagent solution prepared in the same way but which did not contain ABTS, by measuring the activity of the peroxidase at different times. The following Table shows the results obtained:

<table>
<thead>
<tr>
<th>No. of weeks</th>
<th>without ABTS</th>
<th>with ABTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>at 33°C</td>
<td>ABTS</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>35%</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4%</td>
<td></td>
</tr>
</tbody>
</table>

The above results show that, after storage for 3 weeks at 33°C., in the reagent solution according to the present invention, the peroxidase activity was seven to eight times higher than that in a sample only stabilized with azide.

It will be understood that the specification and examples are illustrative but not limitative of the present invention and that other embodiments within the spirit and scope of the invention will suggest themselves to those skilled in the art.

What is claimed is:

1. Reagent composition for the enzymatic determination of glucose which composition comprises glucose oxidase, peroxidase, a chromogen, a buffer, an azide, and 2,2'-azino-di-(3-ethyl-benzothiazoline-6-sulfonic acid).

2. Composition as claimed in claim 1 wherein the 2,2'-azino-di-(3-ethyl-benzothiazoline-6-sulfonic acid) acts as the said chromogen.

3. Composition as claimed in claim 1 wherein the azide is an alkali metal azide.

4. Composition as claimed in claim 1 wherein said buffer is phosphate buffer.

5. Composition as claimed in claim 1 which comprises:
   a buffer, pH 6.2 to 7.5
   2.5 to 15 × 10^3 U glucose oxidase,
   30 to 1000 U peroxidase,
   0.05 to 0.2 grams of an azide, and
   0.25 to 1.5 grams 2,2'-azino-di-(3-ethyl-benzothiazoline-6-sulfonic acid),
   based on each liter of ultimate applied aqueous reagent composition solution.

6. Composition as claimed in claim 1 which comprises:
   a buffer, pH 6.2 to 7.5
   2.5 to 15 × 10^3 U glucose oxidase,
   30 to 1000 U peroxidase,
   0.05 to 0.2 grams of an azide,
   1 to 10 milligrams of 2,2'-azino-di-(3-ethyl-benzothiazoline-6-sulfonic acid), and
   50 to 70 milligrams o-dianisidine,
   based on each liter of ultimate applied aqueous reagent composition solution.

7. Composition as claimed in claim 5 wherein said composition is in solid form.

8. Composition as claimed in claim 5 wherein said composition is in the form of an aqueous solution.

9. Composition as claimed in claim 1 which comprises:
   0.1M phosphate buffer, pH 6.5 to 7.2,
   9 to 12 × 10^3 U glucose oxidase,
   150 to 500 U peroxidase,
   0.1 gram of an azide, and
   0.5 gram 2,2'-azino-di-(3-ethyl-benzothiazoline-6-sulfonic acid),
   based on each liter of ultimate applied aqueous reagent composition solid.

10. Composition as claimed in claim 1 which comprises:
    0.1M phosphate buffer, pH 6.5 to 7.2,
    9 to 12 × 10^3 U glucose oxidase,
    150 to 500 U peroxidase,
    0.1 gram of an azide,
    2 to 5 milligrams 2,2'-azino-di-(3-ethyl-benzothiazoline-6-sulfonic acid), and
    50 to 70 milligrams o-dianisidine,
based on each liter of ultimate applied aqueous reagent composition solution.

11. Composition as claimed in claim 9 wherein said azide is sodium azide.

12. Method for the enzymatic determination of glucose which method comprises contacting a test sample with a reagent composition comprising glucose oxidase, peroxidase, a chromogen, a buffer, an azide, and 2,2′-azino-di-(3-ethyl-benzothiazoline-6-sulfonic acid).

13. Method as claimed in claim 12 wherein the intensity of the colored material formed is measured and the glucose concentration present in the test sample is determined therefrom.