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With amended claims.

(54) Title: BIOLUMINESCENCE REAGENT FORMULATION

(57) Abstract

An aqueous composition containing luciferase, luciferin, a stabilising polyol and buffer, at pH 5.5 to 7.4, has good stability. It can simply be used, in a bioluminescence assay, by adding stronger buffer, to give a pH close to the optimum for the luciferin-luciferase reaction.

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BIOLUMINESCENCE REAGENT FORMULATION

Field of the Invention

This invention relates to a formulation of a bioluminescence reagent containing e.g. firefly luciferase and luciferin.

Background of the Invention

Bioluminescence is used mainly to assay for the presence of live microorganisms or for matter of organic origin, e.g. food residues on a surface. In either case ATP, which can be released from the microorganisms using a suitable extractant, may be detected by measuring the release of light on reaction with firefly luciferase, luciferin and oxygen.

The assay reagents are often formulated as a kit for use by the customer. Firefly luciferase-luciferin reagents do not have good liquid stability, and it has therefore been necessary to supply them in a freeze-dried form. Such a reagent is normally reconstituted as a solution of adequate buffering capacity at a pH close to 7.7-7.8, the optimum range for the bioluminescence reaction. After reconstitution, the luciferase-luciferin reagent has poor stability, especially if it is exposed to temperatures above 15°C.

Many users would find it a great advantage to have a reagent that is much more stable as a liquid. This stable reagent could be supplied either freeze-dried, along with a suitable reconstitution buffer, or as a liquid; the latter would of course avoid altogether the need for freeze-drying.

Summary of the Invention

According to the present invention, an aqueous luciferase-luciferin reagent is formulated with a polyol and at a pH significantly below the optimum for the bioluminescence reaction. It has unexpectedly been found that a reagent with this composition has good stability even at room temperature. Without wishing to be bound by theory, it is likely that the presence of the polyol helps

to stabilise the enzyme, whilst the lower pH reduces the rate of luciferin decomposition.

A freeze-dried luciferin-luciferase reagent is reconstituted, according to a further aspect of the invention, with water and the polyol to the sub-optimum reaction pH, i.e. to form an aqueous reagent of the invention.

Description of the Invention

In order to achieve maximum sensitivity in an ATP assay, it is necessary that the final pH is in the optimal range. For this reason, the novel reagent itself usually contains a low buffering capacity, and the correct assay pH is reached by the subsequent addition of a strong buffer, e.g. Tris-acetate or Tris-tricine, at the desired final pH. This is conveniently achieved in one of three ways:

(1) shortly before use the strong buffer is added to the liquid reagent;

(2) concentrated buffer is added to the samples to be tested;

(3) in the case of microbial assays, the strong buffer is included in the extractant solution.

Whichever method is used, the strong buffer can be provided as a component of a kit which also contains the novel reagent (either in liquid form or freeze-dried) and other materials necessary for the required assays. Such materials may be conventional, and comprise, for example a magnesium salt (which is required for luciferase activity), a bacteriostatic agent such as sodium azide, a thiol-protecting agent such as dithiothreitol, a bulking and protecting protein such as albumin, and a metal chelator such as EDTA. These materials may be formulated with the luciferase and/or luciferin or may be provided separately.

Examples of the strong buffer are given above. The relatively weak, generally acid, buffer is preferably a substance with a pK close to the desired sub-optiumum pH, such as phosphate (pK 6.8), 2-(N-morpholino)ethanesulphonic

acid (pK 6.1), 3-(N-morpholino)propanesulphonic acid (pK 7.2) etc.

The stabilising agent, the polyol, is preferably of limited molecular weight. It may be, for example, a polyglycol having a molecular weight up to 5,000 or 10,000, or a sugar such as trelalose, but is preferably a polyol that is liquid at room temperature such as ethanediol (ethylene glycol) or, most preferably, glycerol. Suitable alternative polyols can be determined by simple experimentation. The formulation preferably comprises up to 25% by volume of the stabilising agent, e.g. 1 to 20% by volume. Higher concentrations may give better long-term stability, but decrease mechanical stability. The latter is an important consideration for liquid reagents which may be agitated during transport and use. Therefore a compromise level of, say, glycerol is preferred.

The sub-optimal reaction pH is 5.5 to 7.4, e.g. 6-7, and usually about 6.8. This provides adequate stability and allows ready conversion to the buffered, optimum pH.

The following Examples illustrate the invention.

Example 1

Luciferase-luciferin reagents were formulated in the following buffer system:

5 mM potassium phosphate pH 6.8
10 mM magnesium acetate
1 mM di-sodium potassium EDTA
0.3 mg/ml bovine serum albumin
0.05% (w/v) sodium azide
0.5 mM dithioreitol
5% (v/v) glycerol

Various samples were prepared which contained different amounts of firefly luciferase and luciferin, but which initially gave approximately the same light output response in the assay.

The assay was performed at 25°C by adding 0.1 ml ATP (2 nM in 0.1 M Tris-acetate pH 7.8) to 0.1 ml of the

reagent in a cuvette and integrating the light for 10 seconds using a Berthold Biolumat LB9500T luminometer.

Reagent samples were stored in amber plastic vials (Nalgene) at 4°C and at 20°C, and were re-assayed after 1 and 2 months, with the results shown in Table 1.

Table 1

Luciferin (mM)	Luciferase (µg/ml)	% retention of activity 4°C		20°C	
		1 month	2 months	1 month	2 months
0.05	12	97	86	85	66
0.1	6.7	90	86	80	62
0.2	3.7	91	81	78	55

In each case, the stability in liquid form is very much better than normal reagents, formulated at pH 7.8 without glycerol. The limiting factor seems to be the stability of luciferin rather than luciferase, as preparations with more enzyme and less substrate are the more stable.

Example 2

Samples of reagent were formulated as in Example 1, with 0.05 mM luciferin and 12 µg/ml luciferase, but with 10 mM magnesium sulphate instead of the acetate salt and with glycerol at 5, 10 and 15% (v/v). These lost less than 5% of their initial activity during 4 weeks storage at 20°C. This increased stability was at the expense of a reduction of the light output.

Example 3

Samples of luciferase-luciferin reagent were prepared with various additives for comparison. The common constituents were:

0.05 mM luciferin
2 µg/ml luciferase
10 mM magnesium sulphate
1 mM di-sodium potassium EDTA
0.3 mg/ml bovine serum albumin
0.05% (w/v) sodium azide
+20 mM buffer
± glycerol

These were assayed, as in Example 1, after 7 weeks at 25°C, with the results shown in Table 2.

Table 2

Buffer/pH	Additive	% Retention of Activity
Tris-tricine pH 7.8	None	2
Tris-tricine pH 7.8	10% glycerol	15
Tris-tricine pH 7.8	20% glycerol	21
Potassium phosphate pH 6.5	20% glycerol	78

This experiment clearly shows the additive beneficial effects of formulating the reagent at low pH and including glycerol.

CLAIMS

1. An aqueous composition containing luciferase, luciferin, a stabilising polyol and buffer, at pH 5.5 to 7.4.

2. A composition according to claim 1, wherein the pH is about 6.8.

3. A composition according to claim 1 or claim 2, wherein the polyol is glycerol.

4. A composition according to any preceding claim, which contains 1 to 25% by volume of the polyol.

5. A composition according to any preceding claim, which additionally contains at least one component selected from magnesium salts, bacteriostatic agents, thiol-protecting agents, bulking and protecting proteins, and metal chelators.

6. A process for preparing a composition according to any preceding claim, which comprises reconstituting a freeze-dried luciferin-luciferase formulation with water, the polyol and the buffer.

7. An assay kit comprising two containers respectively containing a composition according to any of claims 1 to 5 and a strong buffer which, when mixed with the composition, provides a solution buffered at pH 7.5 to 8.5.

AMENDED CLAIMS

[received by the International Bureau on 25 March 1994 (25.03.94);
original claims unchanged; new claims 8 and 9 added (1 page)]

8. A closed container containing a composition according to any of claims 1 to 5.

9. A bioluminescence assay, which comprises introducing luciferase and luciferin from a composition according to any preceding claim and, prior to the assay, adding a strong buffer which, when mixed with the composition, provides a solution buffered at pH 7.5 to 8.5.

INTERNATIONAL SEARCH REPORT

Inter: ıal Application No
PCT/GB 93/02363

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12Q1/66 C12N9/96

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)
IPC 5 C12Q C12N

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)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 04468 (PROMEGA CORPORATION) 19 March 1992	1-6
A	See page 12, line 31-page 15, line 37; page 17, line 19-page 19, line 37; claims:	7
X	BRAIN RESEARCH vol. 154, no. 2 , 13 October 1978 pages 273 - 284 K.KOGURE ET AL. 'A pictorial representation of endogenous brain ATP by a bioluminescent method.' see page 273 - page 274	1,3-6
Y	US,A,4 235 961 (A.T.LUNDIN) 25 November 1980	1-6
A	see column 1 - column 3; claims	7

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

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31 January 1994	18. 02. 94

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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.
Y	DATABASE WPI Week 9106, Derwent Publications Ltd., London, GB; AN 91-041062 & JP,A,2 308 792 ((KIKK) KIKKOMAN CORP.) 21 December 1990 see abstract	1-6
A	DATABASE WPI Week 8817, Derwent Publications Ltd., London, GB; AN 88-117712 & SU,A,1 339 128 ((MOSU) MOSCOW LOMONOSOV UNIV.) 23 January 1986 see abstract	1,3,4,6

INTERNATIONAL SEARCH REPORT

...information on patent family members

Inter ...al Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9204468	19-03-92	AU-A- 8858391	30-03-92
		EP-A- 0553234	04-08-93
US-A-4235961	25-11-80	NONE	