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(54) Title: STABLE FORMULATION OF VIRAL NEURAMINIDASE

(57) Abstract

Aqueous formulations of viruses having neuraminidase activity, such as influenza and parainfluenza viruses, are made thermostable by incorporating a sugar stabilizer, a pH 4-7 buffer, calcium ion, and optionally a nonionic detergent into the formulation.

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STABLE FORMULATION OF VIRAL NEURAMINIDASE

Field of the Invention

This invention relates to a stable, aqueous formulation of enzymatically active viral neuraminidase.

Background of the Invention

Neuraminidase is an enzyme found in many viruses whose role has been postulated to be involved in spread of the virus from cell to cell. Some of the viruses containing this neuraminidase activity include the influenza viruses (types A and B) and the parainfluenza viruses (types 1, 2 and 3). The current assays for this enzyme have employed numerous methods (see Santer et al. Bioch. Biophys. Acta 523:435 (1978); Tuppy et al. FEBS Letters, 3:72 (1969); Myers et al. Anal. Biochem. 101:166 (1980); Warner et al. Biochem. 18:2783 (1979); Potier et al. Anal. Biochem. 94:287 (1979); and Thomas et al. Anal. Biochem. 88:461 (1978)) but all have been hampered by the lack of stability seen in the neuraminidase activity. This has hampered the consistency of assay results within the time of an assay as well as after long-term storage of the virus.

Influenza neuraminidase is thermolabile (see Cabezas et al. Bioch. Biophys. Acta 616:228 (1980)) with the activity being reduced 60% within 10 minutes at 50°C. Other studies have examined the effect of several detergents such as Triton X-100, Tween 20, sodium dodecyl

sulfate, NP-40, N-laurosylsarcosine, salts and cofactors on activity (see Thomas et al. Anal. Biochem. 88:461 (1978); Aitken Eur. J. Biochem. 107:51 (1980); Bottex et al. Lyon Pharm. 27:327 (1976); Bucher et al. The Influenza Viruses and Influenza (E.D. Kilbourne, ed.), 100, Academic Press, New York (1975); and Cabezas et al. Int. J. Biochem. 14:311 (1982)), but none have sought to further the long-term stability of the neuraminidase activity of viruses for use in different assay systems, be they diagnostic and/or for research.

An object of the present invention is to provide an active, stable liquid formulation of viral neuraminidase activity for use in assay systems, be they for diagnostic purposes or for research. Another object of this system is to provide a liquid formulation for neuraminidase activity in viruses having improved stability. Still another object of this invention is to have a liquid formulation permitting storage for a long period of time in a liquid state facilitating storage and shipping of the virus(es) for use in an assay system, be it diagnostic or for research. Another object of this liquid formulation is to provide a liquid system which is resistant to fluctuations in temperature which may be deleterious to the neuraminidase activity and/or the ability of the virus to be infectious. Yet another object of this invention is a liquid formulation of a neuraminidase-containing virus which is easily made and uses readily available chemicals.

It was not appreciated until this invention that a liquid formulation of neuraminidase-containing viruses could be made which retains neuraminidase activity, can be stored for a long term, and which allows for shipment of fragile neuraminidase-containing viruses.

Disclosure of the Invention

The invention is an aqueous formulation having viral neuraminidase activity comprising:

(a) a virus with neuraminidase activity;

(b) a buffer that maintains the pH of the formulation in the range of 4 and 7;

(c) a thermostabilizing amount of a stabilizer selected from the group consisting of polyhydric sugar alcohols, simple sugars, and disaccharide sugars; and

(d) a divalent metal cation required for neuraminidase activity.

Preferably the formulation also includes a sufficient amount of a nonionic detergent to facilitate release of neuraminidase from the viral envelope.

Another aspect of the invention is a method of stabilizing neuraminidase activity in an aqueous formulation containing a virus with neuraminidase activity comprising adding to the formulation a stabilizing amount of a stabilizer selected from the group consisting of polyhydric sugar alcohols, simple sugars and disaccharide sugars.

Modes for Carrying Out the Invention

Influenza virus and other neuraminidase-containing viruses and their methods of preparation are well known. (See, for instance, Hoyle, L., The Influenza Viruses (S. Gard, C. Hallaver and K.F. Meyer, ed.), 32-38, Springer-Verlag, New York (1968); D.J.S. Arora et al. Can. J. Microbiol. 19, 633 (1973); T. Barrett and S.C. Inglis Virology, A Practical Approach (B.W.J. Mahy, ed.), 140, IRL Press, Washington, D.C (1985); and P. Payment and M. Trudel Biotechnology Applications and Research (P.N. Cheremisinoff and R.P. Oudlette, ed.), 436, Technomic Publishing Co., Lancaster (1985).) Included within the scope of influenza virus and other neuraminidase-

containing viruses are the influenza viruses (types A and B) and the parainfluenza viruses (types 1, 2 and 3).

The amount of virus in the formulation will depend upon the source of virus and the intended purpose of the formulation. When the characteristics of the source of virus are known (e.g., a culture of virus having a known activity and viral concentration) and the intended purpose of the formulation is to provide a standard for neuraminidase, the amount of virus will be sufficient to provide a level of activity higher than the sensitivity level of the assay. Correspondingly, when the characteristics of the source of virus are not known (e.g., a clinical sample of a pharyngeal, nasopharyngeal or respiratory secretion), and the intended purpose of the formulation is to provide a specimen for diagnosis of influenza, the amount of virus will be initially unknown.

Examples of the polyhydric sugar alcohols, and simple and disaccharide sugars to be used as stabilizers in the present invention are trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol, the simple sugars glucose and fructose and the disaccharide sucrose. These polyhydric sugar alcohols and simple and disaccharide sugars can be used alone or in a combination. In order to stabilize the activity of the neuraminidase-containing viruses, the sugar stabilizer is added to the formulation in an amount from 0.2 M to 2.1 M, preferably, 0.6 M to 2.0 M.

The organic and inorganic acid buffers to be used in the present invention maintain the pH of the formulation in the range of about 4.0 to 7.0, preferably 5.5 to 6.5, and can be conventional buffers of organic acids and salts thereof such as citrate buffers (e.g. monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g. succinic acid-monosodium succinate mixture, succinic acid-sodium

hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g. tartaric acid-tartrate mixture, tartaric acid- potassium tartrate mixture, tartaric acid-sodium hydroxide mixture etc.), fumarate buffers (e.g. fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate acid-disodium fumarate mixture), gluconate buffers (e.g. gluconic acid-sodium gluconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium gluconate mixture, etc.) oxalate buffers (e.g. oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g. lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.), acetate buffers (e.g. acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.), phosphate buffers (e.g., monosodium phosphate-disodium phosphate mixture, monosodium phosphate-sodium hydroxide mixture, trisodium phosphate-hydrochloric acid mixture, etc.), 2-(N-morpholino)ethanesulfonc acid, [bis-(2-hydroxyethyl)imino]tris(hydroxymethyl)methane, N-2-acetamidoiminodiacetic acid, 1,3-bis[tris(hydroxymethyl)methylamino]propane, piperazine-N,N'-bis(2-ethanesulfonic acid), N-2-acetamido-2-aminoethanesulfonic acid, 3-(N-morpholino)-2-hydroxypropanesulfonic acid, N-N-bis-(2-hydroxyethyl)2-aminoethanesulfonic acid, 3-(N-morpholino)propanesulfonic acid, 2-[tris(hydroxymethyl)methylamino]ethanesulfonic acid, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 3-{[tris-(hydroxymethy)methyl]amino}-2-hydroxypropanesulfonic acid.

Examples of nonionic detergents that may optionally be included in the formulation are the Pluronics, for example, Polysorbate 80 or Polysorbate 20, Triton X-100, NP-40 and the alkyl glucosides, for example, octyl glucoside and nonyl glucoside. When used, these

detergents are present in the range of 0.1 to 10.0% by weight with a preferred range of about 1.0 to 7.0% by weight.

Examples of a divalent metal to be added is calcium ion. The ion may be added in the form of water soluble salts. It should be added at a level between 1 and 20 mM, preferably at 10 mM. There are some indications that this divalent cation is required for the activity of some of the neuraminidases found in viruses and therefore is included to assure maximal activity of the neuraminidase within the viruses (see S.M. Carroll and J.C. Paulson, Arch. Virol. 71, 273, (1982).

The aqueous formulation of this invention is stable for prolonged periods of time. The formulation of this invention may be stored in a liquid state at various temperatures. A preferred storage temperature range is between about 2^{o} and about $8^{o}C$.

The following examples illustrate the present invention, but are not to be construed to limit the scope of the invention in any manner.

Example 1

Influenza virus type A was put in the liquid formulation/excipient system after growth in a cell culture system and initial purification through clarification of the cell culture media. It was put at a level 10 - 100 times the sensitivity of a modified fluorescent assay procedure (see Kiyotani and associates Zbl. Bakt. Hyg. A. 260, 273, (1985); and T.G. Warner and J.S. O'Brien Biochemistry 18, 2783 (1979)) into: 50 mM acetic acid-sodium hydroxide mixture; 10 mM $CaCl_2$, 0.6 to 2.1 M of stabilizer (as shown in Tables 1 and 2), and a sufficient quantity of deionized water. The acetate buffer maintained the pH at 5.9. This liquid formulation/ excipient system was found to exhibit an increased stability at $40^{o}C$ over the length of the study when

compared to the same level of influenza A virus without the added stabilizer.

As seen in Table 1, the loss of neuraminidase activity reflected in the rate constants indicates an approximate 4- to 30-fold increased level of stabilization over the control Influenza A virus with no added stabilizer. These changes are reflected in the rate constant which is the slope of the line resulting from the plot of the logarithm of the loss of neuraminidase activity of the liquid formulation/excipient system versus time. Enzyme activity was measured using the general fluorometric procedure described by Kiyotani et al., supra. The stability of the liquid formulation/excipient system was considerably greater than that of the control virus without the added stabilizer.

Table 1

Comparative Stability of Neuraminidase Activity of Influenza A Virus with and without Added Stabilizers[1]

Virus Type	Study (days)	Rate Constant (k)	Relative Stability (days)[2]
Control Virus/ Influenza A Type	52	-0.1870	1.00
+ Glucose (1.5 M)	52	-0.0137	13.65
+ Xylitol (2.1 M)	52	-0.0174	10.75
+ D-Sorbitol (1.5 M)	52	-0.0109	17.16
+ Sucrose (1.5 M)	52	-0.0063	29.68
+ D-Mannitol (0.6 M)	52	-0.0466	4.01

1 Based on accelerated 40°C data.

2 A comparison of the relative stability based on the neuraminidase activity of the influenza virus with the control virus (i.e. no added polyhydric sugar alcohols or other sugars) arbitrarily being set at 1.

A similar comparative study was also carried out with influenza B virus. Once again, as seen in Table 2, the loss of neuraminidase activity was greater for the control virus without the added stabilizer than the liquid formulation/excipient system of this invention. Table 2 also shows the stability was increased 2 to 43-fold over that of the control influenza B virus with no added stabilizer.

Table 2

Comparative Stability of Neuraminidase Activity of Influenza B Virus with and without Added Stabilizers[1]

Virus Type	Study (days)	Rate Constant (k)	Relative Stability (days)[2]
Control Virus/ Influenza B Type	54	-0.2114	1.00
+ Glucose (1.5 M)	54	-0.0229	9.23
+ Xylitol (2.1 M)	54	-0.0186	11.37
+ D-Sorbitol (1.5 M)	54	-0.0169	12.51
+ Sucrose (1.5 M)	54	-0.0049	43.14
+ D-Mannitol (0.6 M)	54	-0.1006	2.10

1 Based on accelerated 40°C data.

2 A comparison of the relative stability based on the neuraminidase activity of the influenza virus with the control virus (i.e. no added polyhydric sugar alcohols or other sugars) arbitrarily being set at 1.

Modifications of the above-described modes for carrying out the invention that are obvious to those of skill in virology, chemistry, biochemistry and related fields are intended to be within the scope of the following claims.

Claims

1. An aqueous formulation having viral neuraminidase activity comprising:

(a) a virus with neuraminidase activity;

(b) a buffer that maintains the pH of the formulation in the range of 4 and 7;

(c) a thermostabilizing amount of a stabilizer selected from the group consisting of polyhydric sugar alcohols, simple sugars, and disaccharide sugars; and

(d) a divalent metal cation required for neuraminidase activity.

2. The formulation of claim 1 wherein the formulation includes:

(e) a nonionic detergent.

3. The formulation of claim 1 or 2 wherein the divalent metal cation is calcium ion.

4. The formulation of claim 3 wherein the stabilizer is present at a concentration of 0.2 M to 2.1 M and the calcium ion is present at a concentration of 1 to 20 mM.

5. The formulation of claim 1, 2, 3 or 4 wherein the buffer maintains the pH between 5.5 and 6.5.

6. The formulation of claim 1, 2, 3, 4 or 5 wherein the buffer is selected from the group consisting of citrate, succinate, tartarate, fumarate, gluconate, oxalate, lactate, acetate, phosphate buffers and 2-(N-

morpholino)ethanesulfonc acid, {bis-(2-hydroxy-ethyl)imino}tris(hydroxymethyl)methane, N-2-acetamido-iminodiacetic acid, 1,3-bis{tris(hydroxymethyl)methyl-amino}propane, piperazine-N,N'-bis(2-ethanesulfonic acid), N-2-acetamido-2-aminoethanesulfonic acid, 3-(N-morpho-lino)-2-hydroxypropanesulfonic acid, N-N-bis-(2-hydroxy-ethyl)2-aminoethanesulfonic acid, 3-(N-morpholino)propane-sulfonic acid, 2-{tris(hydroxymethyl)methylamino}ethane-sulfonic acid, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 3-{{tris-(hydroxymethy)methy}amino}-2-hydroxypropanesulfonic acid.

7. The formulation of claim 1, 2, 3, 4, 5 or 6 wherein the stabilizer is selected from the group consisting of glycerin, erythritol, arabitol, xylitol, sorbitol, mannitol, glucose, fructose and sucrose.

8. The formulation of claim 7 wherein the formulation includes 0.1 to 10% by weight of a nonionic detergent, the divalent metal cation is calcium ion and is present at 1 to 20 mM, the stabilizer is present at 0.2 to 2.1 M, and the buffer maintains the pH at 5.5 to 6.5 and is selected from the group consisting of citrate, succinate, tartarate, fumarate, gluconate, oxalate, lactate, acetate, phosphate buffers and and 2-(N-morpho-lino)ethanesulfonc acid, {bis-(2-hydroxy-ethyl)imino}tris(hydroxymethyl)methane, N-2-acetamido-iminodiacetic acid, 1,3-bis{tris(hydroxymethyl)methyl-amino}propane, piperazine-N,N'-bis(2-ethanesulfonic acid), N-2-acetamido-2-aminoethanesulfonic acid, 3-(N-morpho-lino)-2-hydroxypropanesulfonic acid, N-N-bis-(2-hydroxy-ethyl)2-aminoethanesulfonic acid, 3-(N-morpholino)propane-sulfonic acid, 2-{tris(hydroxymethyl)methylamino}ethane-sulfonic acid, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 3-{{tris-(hydroxymethy)methy}amino}-2-hydroxypropanesulfonic acid.

9. The formulation of claim 1, 2, 3, 4, 5, 6, 7 or 8 wherein the virus is an influenza virus.

10. The formulation of claim 9 wherein the influenza virus is influenza virus A.

11. The formulation of claim 9 wherein the influenza virus is influenza virus B.

12. The formulation of claim 9 wherein the virus is a parainfluenza virus.

13. The formulation of claim 12 wherein the parainfluenza virus is parainfluenza 1.

14. The formulation of claim 12 wherein the parainfluenze virus is parainfluenza 2.

15. The formulation of claim 12 wherein the parainfluenza virus is parainfluenza 3.

16. A method of stabilizing neuraminidase activity in an aqueous formulation containing a virus with neuraminidase activity comprising adding to the formulation a stabilizing amount of stabilizer selected from the group consisting of polyhydric sugar alcohols, simple sugars and disaccharide sugars.

17. The method of claim 16 wherein a pH 4-7 buffer, calcium ion and a nonionic detergent are also added to the formulation.

18. The method of claim 16 or 17 wherein the stabilizer is selected from the group consisting of

glycerin, erythritol, arabitol, xylitol, sorbitol, mannitol, glucose, fructose and sucrose.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/07680

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

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

IPC(5): C12N 7/00
U.S. CL: 435/188, 200, 235.1, 260, 963; 424/89, 93; 436/18, 176

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/188,200,235.1,260,963; 424/89,93; 436/18,176

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US ,A, 4,356,169 **(SIMONS ET AL.)** 26 October 1982, see the entire document.	1-18
Y	US , A, 4,147,772 **(McALEER ET AL.)** 03 April 1979, see the entire document.	1-18
Y	US , A, 3,961,046 **(CERINI)** 01 June 1976, see the entire document.	1-18
Y	US , A, 4,537,769 **(CERINI)** 27 August 1985, see the entire document.	1-18
Y	US , A, 3,927,209 **(STRAUB)** 16 December 1975, see the entire document.	1-18
A	**Biochimica et Biophysica Acta,** Vol. 616, issued 1980, **CABEZAS ET AL.**, "Neuraminidase from Influenza Virus A (H3N2). Specificity Towards Several Substrates and Procedure of Activity Determination", pages 228-238, see the entire document.	1-18

* Special categories of cited documents: [10]

"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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"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

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report [14]
27 March 1991	19 APR 1991

International Searching Authority	Signature of Authorized Officer
ISA/US	J.M. Stone (vsh)

Form PCT/ISA/210 (second sheet) (July 1989)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	**Biological Abstracts**, Volume 63, No. 7, issued 01 April 1977, **BAKER ET AL.**, "Effect of CA# on the stability of influenza virus neuraminidase", see pages 4163-4164, abstract no. 42287, Arch Virol., 52(1/2), 7-18.	1-18

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4 ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.