Patented Jan. 6, 1953

2,624,691

UNITED STATES PATENT OFFICE

2,624,691

FIBRINOLYSIN DERIVED FROM BLOOD AND METHODS OF OBTAINING THE SAME

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No Drawing. Application April 22, 1946, Serial No. 663,952

5 Claims. (Cl. 195—62)

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The invention relates to new and useful purified pro-fibrinolysin and fibrinolysin products derived from blood serum and to methods for obtaining the same. More particularly the invention relates to new pro-fibrinolysin and fibrinolysin products which are entirely free from prothrombin, thrombin, fibrin, fibrinogen and other undesired products present in, or derived from, blood.

It is known that a factor, called "lysin factor," exists in blood or blood serum or plasma which is capable, in the presence of another factor produced during growth of hemolytic streptococci, of dissolving fibrin.

The inactive lysin factor in plasma or serum is apparently an inactive lytic enzyme, or profibrinolysin, which is activated by the presence of the streptococcal activator or co-factor to provide a fibrinolytic product capable of dissolving blood clots or fibrin.

It is also known that, instead of using streptococcal activator, the inactive prolysin factor derived from blood or plasma or serum can be activated by treatment with various organic solvents and compounds to give a crude fibrinolytic product. However, regardless of the activator used, such prior fibrinolytic and pro-fibrinolytic products have been contaminated by thrombin, anti-thrombin, thromboplastin, prothrombin and other undesirable proteins and impurities and the final fibrinolytic products have only had a low degree of dissolving power for blood clots and fibrin. A further disadvantage of previously prepared fibrinolytic products has been that they have activated prothrombin so that it is converted to thrombin, which latter exercises a clotting action on fibrinogen.

It is an object of the present invention to provide new improved profibrinolytic and fibrinolytic products which are highly active and entirely free from fibrin, fibrinogen, thrombin, thromboplastin and prothrombin. Another object is to provide such products which may or may not contain anti-thrombin. A further object is to provide methods whereby such new and improved products can be obtained by simple and direct procedures.

The above mentioned and other objects of the invention are realized by carrying out a carefully controlled salt precipitation fractionation of a special mammalian blood serum from which all prothrombin and thrombin, and preferably also fibrin and fibrinogen, have already been removed by methods having little or no destructive action on the profibrinolytic substances contained in

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the original serum and, if desired, subsequently activating the profibrinolytic product to impart fibrinolytic properties to it.

In obtaining the new products of the invention one can start with a serum which is essentially a blood plasma from which prothrombin has been removed or with a serum from which fibrinogen and fibrin, as well as prothrombin and thrombin, have been removed. In either case, a salt precipitation step is first used which removes solid protein impurities. In the case of the plasma from which prothrombin has been removed, the first fractionation step brings down and removes the fibrinogen as well as impurities. In the second salt precipitation step the solid profibrinolysin product comes out and is separated from soluble impurities. Both salt precipitation steps must be used under carefully controlled conditions and are only satisfactory when the starting materials are as specified.

The serum above mentioned from which fibrin and fibrinogen, as well as prothrombin and thrombin, have been removed is a new product of particular usefulness in the present invention and is made by special procedures which are described more fully hereinafter.

The invention can be illustrated by the following examples.

EXAMPLE 1.—PREPARATION OF SPECIAL SERUM

A 5 gallon drum of frozen plasma oxalated with a known anticoagulant quantity and proportion of oxalic acid and sodium oxalate as described in U. S. Patent No. 2,394,566 of Smith and Seegers, issued February 12, 1946, is permitted to stand at room temperature (24 to 26° C.) for 24 hours after which the remaining unmelted portion is broken up with an ice pick and a stainless steel warming coil containing running warm water at about 40° C. is inserted into the mixture and the mixture stirred. The remaining frozen material is rapidly melted. The warming is then continued with vigorous agitation.

When the temperature of the plasma reaches about 5 to 8° C., the calculated quantity of calcium chloride solution is added in amount which is from 0.2 to 0.3% in excess of that needed to react with and precipitate the anticoagulant. The temperature of the plasma is allowed to rise to about 24° C. At 18 to 24° C. strands of fibrin begin to appear and the vigor of stirring is increased to prevent a gel of fibrin from forming. Stirring is continued for 30 minutes after the fibrin is whipped out to allow for complete conversion of all prothrombin to thrombin and for

the anti-thrombin to completely destroy all thrombin. At the end of this time the stirring is stopped, the fibrin allowed to rise to the surface and the clear serum siphoned off.

If, through failure to stir with enough vigor, a gel forms instead of strands of fibrin, when the temperature reaches about 18° C., one can also obtain the serum separate from the fibrin by working and kneading the gel in a cheese cloth bag while draining off the clear serum. However, this method is time-consuming and it is preferred to prevent gel formation by very vigorous stirring of the mixture.

The clear serum of this example is an amber liquid free from prothrombin, thrombin, fibrinogen and fibrin. It contains profibrinolysin and is excellently suited to further purification by salt precipitation fractionation, as given in Example 2 below.

That the serum of this example contains no fibrinogen, prothrombin or thrombin is evident from the following tests:

Tests

1. One ml. serum+0.1 ml. of 100 U Thrombin→no clot. Therefore all fibrinogen removed.

2. 1 ml. purified fibrinogen+0.2 ml. serum→no clot. Therefore all thrombin destroyed.

3. 1 ml. purified fibrinogen+1 ml. serum+0.1 ml. Purified Lung Extract (9)→no clot. Therefore all prothrombin converted to thrombin and destroyed as proved in 2.

EXAMPLE 2.—PREPARATION OF PROFIBRINOLYSIN FROM SPECIAL SERUM

The special serum from Example 1 is brought to a temperature of about 4 to 6° C. (preferably 5° C.) and saturated ammonium sulfate solution added drop by drop with constant stirring to about 24 to 26% of saturation (preferably 25%). The precipitated protein impurities are then centrifuged off and the supernatant brought to about −1° C. to +1° C. (preferably °0 C.). The degree of its saturation is then brought to about 28 to 31% of saturation (preferably 29%) by further addition of ammonium sulfate solution with stirring. This further degree of saturation precipitates the profibrinolysin which is collected by centrifugation and separated from soluble impurities. By washing the profibrinolysin several times with ammonium sulfate solution of a strength which is 29% of saturation a practically white solid is obtained which can be freeze-dried (frozen and dried under reduced pressure) to give a dry, white, product containing purified profibrinolysin free from thromboplastin, prothrombin, thrombin, fibrinogen and fibrin. If the washing is omitted, a satisfactory product is nevertheless obtained, although it will probably contain some anti-thrombin. However, the anti-thrombin does not interfere with the fibrinolytic action of the final product because anti-thrombin destroys thrombin.

EXAMPLE 3.—PREPARATION OF FIBRINOLYSIN FROM PROFIBRINOLYSIN

In spite of the fact that all prothrombin, thrombin, fibrinogen and fibrin have been removed from the new profibrinolysin of this invention, it has been found that the new profibrinolysin product can be activated by streptococcal activator and by the organic solvents, especially chloroform, and other organic compounds and products known to be useful in activating the crude profibrinolysin products of the prior art. The following procedure illustrates the activation.

The profibrinolysin from Example 2 is dissolved in 500 ml. of distilled water, the solution transferred to a separatory funnel and shaken intermittently for 30 minutes with 100 ml. of chloroform. After this time the chloroform layer is separated and the aqueous phase dialyzed for 16 hours against cold running tap water. The dialysis produces a precipitate of fibrinolysin which is collected and separated by centrifugation. The fibrinolysin product is dissolved in 500 ml. of saline (0.85%) and diluted to 7500 ml. with cold distilled water. The solution is cooled to 0° C. and adjusted to pH 5.5 with N HCl. A precipitate of fibrinolysin results and is collected by centrifugation while the supernatant with its impurities is discarded. The acid salting out step at pH about 5.5 can be repeated if desired or the precipitate can be directly taken up in 500 ml. of physiological saline and adjusted to pH 7, shell frozen in a flask and lyophilized (dried by vacuum desiccation of the frozen material) to give a stable, purified and potent, water soluble, saline soluble fibrinolytic product which does not clot fibrinogen and does not convert prothrombin to thrombin, but which does destroy prothrombin, fibrinogen and fibrin and is not inhibited by prothrombin.

An advantage of the present invention is that it makes available for the first time a reliable method of assay for fibrinolysin because it provides a purified system not heretofore known. Thus, one unit of fibrinolysin is that amount which will dissolve 1 ml. of a 0.3% fibrin clot (e. g. that made by adding thrombin to a fibrinogen solution) in 120 seconds at pH 7.2 and 45° C. in an isotonic saline system buffered with imidazole (Mertz et al., Proc. Soc. Exp. Biol. & Med. 41, 657 (1939)).

The method of assay is as follows. A 0.2 ml. sample of a saline solution of fibrinolysin containing a known weight of fibrinolysin is added to 0.1 ml. of 100 unit thrombin in a 10 x 75 mm. test tube. 0.3 ml. of a 0.3% fibrinogen solution containing imidazole buffer is then blown into the test tube and a stop watch started simultaneously. The tube is then placed in a water bath at 45° C. and removed every 15 seconds and tilted gently. The end point of the assay is the earliest time when the tube contents flow upon gentle tilting of the tube.

Using the above described method of assay it is found that the fibrinolysin product of Example 1 has a strength of about 30 units per milligram of nitrogen. Fibrinolysin products and their corresponding unactivated profibrinolysin products are consistently obtainable with a potency for the fibrinolysin product of more than about 5 units per milligram of nitrogen up to about 30 units per milligram of nitrogen. By following the methods of the prior art for obtaining fibrinolytic products potencies much less than 5 units per milligram of nitrogen are obtained. Thus, the present invention provides an improvement in potency ranging from several hundred per cent up to about six or eight thousand per cent, in addition to providing a product entirely free from prothrombin, thrombin, fibrinogen and fibrin.

Although as shown above, the preferred starting material for salt precipitation fractionation is the special serum from which fibrinogen and fibrin have been removed, one can also use the special serum or plasma containing fibrinogen but

from which all prothrombin has been removed. The reason for this is that the first salt precipitation step of the new method not only eliminates certain impurities common to both varieties of the serum, but also removes all fibrinogen present in the one which contains it, thus giving in the first step practically the same product whether using the serum from conversion of prothrombin to thrombin, followed by destruction of the latter by antithrombin, or using the serum from which prothrombin itself is removed without change. This is illustrated by the following example which utilizes a serum consisting of plasma from which prothrombin as such has been removed.

EXAMPLE 4.—PROFIBRINOLYSIN AND FIBRINOLYSIN FROM SERUM NOT CONTAINING PROTHROMBIN

4,000 ml. of beef plasma are stirred while gradually adding 600 ml. of magnesium hydroxide cream prepared by dispersing 500 gr. of hydromagma paste in 1,000 ml. of distilled water. The concentrated paste is made by adding slowly and with stirring 5 liters of U. S. P. ammonium hydroxide (concentrated) to 20 liters of 20% magnesium chloride solution, allowing the precipitate to settle, decanting the supernatant and washing the precipitate several times with water to remove excess ammonia and thereafter centrifuging the washed precipitate to obtain a packed concentrated magnesium hydroxide paste.

The magnesium hydroxide with its adsorbed prothrombin is removed by centrifugation in a bronze baskethead on a centrifuge revolving at 5,000 R. P. M. for 10 minutes. The clear supernatant liquid is removed and cooled to 5° C. whereupon saturated ammonium sulphate solution is added dropwise with constant stirring until 25% of saturation is attained. The precipitated proteins, consisting largely of fibrinogen, are removed from the mixture by centrifugation, the supernatant cooled to 0° C. and the saturation increased to 29% by the further addition of saturated ammonium sulphate solution with continuous stirring. As described above under Example 2, this further degree of saturation precipitates the profibrinolysin which is then collected by centrifugation and separated from soluble impurities. The profibrinolysin thus prepared can then be activated to give fibrinolysin as described under Example 3.

The following tables show the activities of various fibrinolysin products when prepared by the methods of Example 3 (Table 1—using serum from which prothrombin is removed by coagulation) and Example 4 (Table 2—using serum from which prothrombin is removed by adsorption).

Table 1.—Preparations of fibrinolysin using serum from which prothrombin is removed by coagulation

Run No.	Protein nitrogen percent	U./mg. (units of fibrinolysin per milligram of sample used)	U./mg. N (units of fibrinolysin per milligram of protein nitrogen in sample used)
205	11	2.03	18.45
206	8	2.07	25.89
207	11	2.03	18.45
211	10	2.03	20.30
304	8	1.77	22.13
305	11	2.53	23.00
306	9	2.54	28.22

Table 2.—Preparations of fibrinolysin using serum from which prothrombin is removed by adsorption

Run No.	Protein nitrogen percent	U./mg. (units of fibrinolysin per milligram of sample used)	U./mg. N (units of fibrinolysin per milligram of protein nitrogen in sample used)
128	9	0.37	4.11
112	3	.23	7.19
005	6	.03	4.75

Although the data of the above tables indicate the superiority of starting with a serum prepared by the coagulation method, it should be understood that the product made by using a serum from which prothrombin has been removed by other methods, such as adsorption on magnesium hydroxide, are satisfactory fibrinolytic products for use in dissolving fibrin and blood clots generally even though they are of lower potency than products made by using the other starting material, since they do not contain any prothrombin, thromboplastin, thrombin, or fibrinogen and any protein impurities present do not deleteriously affect the fibrinolytic product or its desirable functions. All of the fibrinolytic products of this invention are useful wherever it is desired to dissolve fibrin without damaging body tissues. For example, the new products are useful for removal of blood clots on or in the body, the removal of the crusts or scabs from sores, eschars from burned surfaces, and for debridement to remove damaged and dead tissues, for example during preparation of body surfaces for skin grafting.

In the above examples ammonium sulphate is used as a precipitating salt, but any protein precipitating water soluble salt, such as an alkali metal or ammonium sulphate or like water soluble salt, can also be used.

The examples show the preferred use of ammonium sulfate with temperatures between about 4 and 6° C. at 24 to 26% sulfate concentration for the first precipitation step and temperatures between about +1° C. and −1° C. at 28 to 31% sulfate concentration for the second precipitation step. However, it will be obvious to those skilled in the art that these conditions will vary if one replaces ammonium sulfate with some other water soluble protein precipitating salt. Furthermore, when using a salt such as ammonium sulfate, one can use temperatures as low as about −5° C. and as high as about 10° C. for both precipitations and still get good results, providing the optimum concentrations of salt for precipitating protein impurities without precipitation of prolysin are used in the first step and the optimum concentrations of salt for precipitating the prolysin without precipitating impurities are used in the second step. In general, salt concentrations for either step will be those corresponding to more than about 20% of saturation with ammonium sulfate and less than about 35% of saturation with ammonium sulfate. In view of the new method of assay given above, it is an easy matter in a given instance to determine these optimum concentrations. The limiting temperature of about −5° C. is determined by the fact that freezing will occur at temperatures substantially lower than this while the limit of about +10° C. is determined by the tendency of the products to become denaturated at higher temperatures.

In general, therefore, temperatures above freezing of the serum are used which cause no substantial denaturation of profibrinolysin and related protein, and concentrations of precipitating salt are used which, in the first step, will be as high as possible without throwing out any substantial amount of profibrinolysin from solution and which, in the second step, are of the optimum value for precipitating out profibrinolysin while leaving impurities in solution.

Although serums derived from bovine blood are used in the examples, any other serum from mammalian blood, such as human blood, can be used in accordance with the present invention to obtain comparable results.

What I claim as my invention is:

1. Method for obtaining profibrinolysin which comprises treating serum of the class mammalian blood serum and mammalian blood serum containing thromboplastin and fibrinogen but no prothrombin, with a protein precipitating water soluble sulfate salt, at temperatures above freezing for said serum and below that causing protein denaturation, at a maximum concentration of said salt for precipitating protein above that corresponding to about 20 percent of saturation with ammonium sulfate and below that corresponding to about 35 percent of saturation with ammonium sulfate not causing substantial precipitation of profibrinolysin contained therein, removing the solution containing profibrinolysin from precipitated proteins, increasing the concentration of said water soluble sulfate salt in the solution containing profibrinolysin to a dgree optimal for precipitation of profibrinolysin with minimum precipitation of impurities and within the above limits of corresponding ammonium sulfate saturation, and separating the precipitated profibrinolysin from the solution.

2. Method for obtaining fibrinolysin which comprises obtaining profibrinolysin according to claim 1 activating it in aqueous solution with chloroform, separating the chloroform, dialyzing the activated aqueous solution thereby precipitating fibrinolysin and removing the precipitated fibrinolysin.

3. Method for obtaining profibrinolysin which comprises treating mammalian blood serum free from prothrombin, thrombin, fibrin and fibrinogen with ammonium sulfate salt, at about 5° C. and until a concentration of about 25 percent of saturation with ammonium sulfate is attained, removing the solution containing profibrinolysin from the resulting precipitated proteins, increasing the concentration of ammonium sulfate in the profibrinolysin solution to about 29 percent of saturation and lowering the temperature of the solution to about 0° C. to precipitate profibrinolysin and separating the precipitated profibrinolysin from the solution.

4. Method for obtaining fibrinolysin which comprises obtaining profibrinolysin according to claim 3, activating it in aqueous solution with chloroform, separating the chloroform, dialyzing the active aqueous solution thereby precipitating fibrinolysin and removing the precipitated fibrinolysin.

5. Fibrinolysin derived from mammalian blood, said fibrinolysin having a potency of 5 to 30 units per milligram of nitrogen; being soluble in water and saline, free from prothrombin, thrombin, thromboplastin, fibrinogen and fibrin; incapable of clotting fibrinogen; incapable of converting prothrombin to thrombin; not inhibited by prothrombin; capable of destroying prothrombin, fibrinogen and fibrin; and said fibrinolysin in aqueous solution remaining in solution without clot formation when treated with any one product of the class consisting of thrombin, fibrinogen, and a combination of fibrinogen and thromboplastin.

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