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METHOD OF PURIFYING LABILE ENZYMES CONTAMINATED WITH HEMOLYSIN-O

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1 The present invention is concerned with a new method of purifying enzymes. More particularly the present invention is concerned with a method of removing soluble hemolysins from a mixture containing beneficial enzymes.

In recent years considerable interest has developed in the production of beneficial enzymes by the fermentation of various bacteria such as Clostridium welchii, various species of streptococci, and even some species of staphylococci. These enzymes are useful in many phases of medicine, for instance in the liquefaction of purulent exudates, in the lysis of blood clots or in the lysis of other material, depending upon the enzyme employed. However, in bacterial fermentations which produce such enzymes, a harmful hemolysin is also produced in most instances. Hemolysins have the dangerous property of hemolyzing red blood cells and also often have a cardiotoxic property which causes death if the hemolysin is injected into the blood stream of an animal in any appreciable quantity. Therefore, the hemolysin must be removed, inactivated or neutralized before the beneficial enzymes can be employed with complete safety.

The properties of hemolysins produced by bacteria of the above types are fairly well known and have been reported upon in numerous literary articles, for instance see "Journal of Immunology," vol. 38 (pages 283—300), and "Journal of Pathology and Bacteriology," vol. 49 (pages 49—51). A hemolysin which is known as hemolysin-O, or streptolysin-O if formed by streptococci, is sometimes produced when bacteria are grown on blood serum but in the production of enzymes, a fermentation media containing blood serum is rarely if ever employed and, therefore, this type of hemolysin is not a major source of trouble. When grown on fermentation media free of blood serum bacteria of the above types produce an oxygen-labile hemolysin which is referred to in this specification and claims as hemolysin-O or streptolysin-O if produced by streptococci, and it is with the removal of this type of hemolysin from a mixture of enzymes that this invention is concerned. As indicated by its name, hemolysin-O is capable of existing in an oxidized and a reduced form. When in the reduced form, the hemolysin is converted to the oxidized form by oxidation. It may be reconverted to the reduced form by a sulfhydryl reducing agent such as thioglycollic acid, cysteine or glutathione, or by other reducing agents such as sodium bisulfite. When in the oxidized form the hemolysin ordinarily does not display its typical hemolytic properties, but it must be remembered that the animal body may contain cysteine which will reduce the hemolysin to the reduced form and thus if the oxidized form is injected into a living organism it may result in hemolysis. For this reason hemolysin-O should be removed or inactivated regardless of whether it is in the reduced or oxidized form.

Many substances have been reported to inactivate hemolysin-O including the lower alcohols, acetone, chloroform, and certain steroids including cholesterol. In any proposed procedure for removing or neutralizing this hemolysin from a mixture of enzymes, however, it must be remembered that enzymes are very labile compounds and in removing or neutralizing the hemolysin, a procedure must be employed which does not inactivate or destroy the enzymes. Most of the above reagents, when used under conditions necessary for the destruction of the hemolysin, also destroy or deactivate the desired enzymes and for this reason they cannot be employed in an enzyme purification process.

It has now been found that, by the procedure of this invention, cholesterol can be successfully employed to remove the harmful hemolysin-O from a mixture of enzymes without destruction or permanent inactivation of the desired enzymes. It has been previously reported, that under certain conditions, cholesterol will completely inactivate hemolysin-O but the conditions required are not satisfactory for an enzyme purification procedure. According to the procedure of this invention, no attempt is made to completely neutralize the hemolysin, but rather an insoluble hemolysin-cholesterol complex is formed at a very low temperature and this complex is then separated by mechanical means.

Cholesterol, as is well known, is only very slightly soluble in cold water and an aqueous mixture of beneficial enzymes containing a large amount of a harmful hemolysin cannot be purified satisfactorily by simply dissolving cholesterol in an aqueous medium containing the enzymes. High temperatures cannot be employed to obtain greater solubility of the cholesterol because such temperatures will inactivate the enzymes. An organic solution of the enzymes cannot readily be prepared and this solution treated with cholesterol for such a strong concentration of organic solvent also tends to deactivate the desired enzymes. Thus it may be seen that the purification of a mixture of enzymes with cholesterol involves a great many difficulties.

The new procedure of this invention, which
overcomes the above difficulties, broadly comprises adding to an aqueous solution of the enzyme containing material, a quantity of water miscible organic liquid protein precipitant at least sufficient to result in the precipitation of the desired enzyme or enzymes from said solution, said organic liquid having dissolved therein an amount of cholesterol at least sufficient to form a complex with substantially all of the hemolyas in said solution of enzyme containing material; removing the resulting precipitate comprising enzyme material, hemolyasin-cholesterol complex and unreacted cholesterol; extracting this precipitate with an aqueous solvent to dissolve the enzyme material; and removing the remaining insoluble cholesterol-hemolyasin complex and free cholesterol from the reconstituted solution by, for instance, filtration or centrifugation. The removal of the cholesterol-hemolyasin complex is necessary because this complex retains much of the hemolytic activity of the uncombined hemolyasin.

The solution of enzyme material to be treated by a new process of this invention may comprise the unmodified fermentation medium or it may comprise fermentation medium which has been previously treated in various ways for various purposes. For instance the new process of this invention may employ a fermentation medium which has been treated to remove the microorganisnal debris. On the other hand, the solution of enzymes to be treated by the process of this invention may comprise a reconstituted solution of enzyme material. For instance the enzyme material may be precipitated from the fermentation medium, purified by various procedures such as alcoholic fractionation, an aqueous concentrate prepared from the purified material, and this concentrate employed in the process of this invention. In other words, any aqueous solution of enzymes containing hemolyasin-o can be satisfactorily purified by the new process of this invention.

The cholesterol should be employed in a minimum amount of .01 mg. of cholesterol for each 10,000 Bernheimer (Journal of General Physiology 36, 237-283) units of hemolyasin. If too small a quantity of cholesterol is employed, it results in incomplete removal of the yas. As the assay for hemolyasin-o is not accurate to within close limits, it is usually advantageous to employ at least .05 mg. of cholesterol per each 10,000 Bernheimer units. An excess of cholesterol is not unduly detrimental and amounts as high as 80 mg. of cholesterol per 10,000 units of hemolyasin may be employed with satisfactory results. In fact, tests have been made where cholesterol concentrations as high as 1,000 mg. of cholesterol for each 10,000 Bernheimer units of hemolyasin were employed but such high concentrations are not ordinarily employed for reasons of economy. The optimum amount of cholesterol is usually between 0.2 and 2.0 mg. of cholesterol per 10,000 units of hemolyasin.

Practically any water miscible organic solvent for cholesterol which is also a protein precipitant may be employed. The most suitable examples of such solvents are the lower aliphatic alcohols but other solvents such as acetone and ether may also be employed. Ethyl alcohol is the preferred protein precipitant for reasons of economy and convenience and because cholesterol is relatively soluble therein. If methyl alcohol is employed, difficulty may be encountered in obtaining complete solution of the calculated amount of cholesterol in the desired amount of solvent, but this difficulty may be overcome by the addition to the methyl alcohol of other solvents having greater solvent power for cholesterol. For instance ethyl ether may be added to the methyl alcohol until the cholesterol dissolves or alternatively the cholesterol may be first dissolved in ether or the like and this solution added to the methyl alcohol. Of course, the above procedure may also be employed to increase the solubility of the cholesterol in organic solvents other than methyl alcohol in which cholesterol is not readily soluble.

The procedure for protein precipitation by organic solvents is well known to those skilled in the art and as enzymes are protein materials the general rules of the art may be employed in choosing an organic solvent and in determining a minimum amount of the organic solvent necessary for precipitation of the enzymes from an aqueous solution. Of course, a minimum amount of organic solvent may be empirically determined, if desired, by assay of the supernatant after addition of a given amount of precipitant. For instance the calculated amount of cholesterol may be dissolved in the minimum amount of organic solvent necessary for its solution and this added to the aqueous protein mixture. If a sample of the supernatant liquid then shows substantial enzymatic activity on assay, additional organic solvent can be added to obtain complete precipitation. Since hemolyasin-o is a protein material and will be precipitated by the organic solvent, it will be obvious to those skilled in the art that in an empirical method such as the above, the quantity of organic solvent containing the calculated amount of cholesterol should be added prior to the addition of any organic solvent which does not contain cholesterol as otherwise the hemolyasin might be at least partially precipitated and intimate contact of all of the hemolyasin with the cholesterol would not be obtained.

It should be remembered, however, that the above is for the purpose of determining a minimum amount of organic solvent necessary under a given set of conditions and a large excess of organic solvent can be satisfactorily employed in the process of this invention. In fact an amount of organic solvent sufficient to give a resulting mixture having an organic solvent concentration as high as 70%, or even 80%, can readily be employed. Of course, a large excess of organic solvent is not ordinarily employed for reasons of economy.

The organic solution of cholesterol should be added slowly to the aqueous solution of enzymes. There are two reasons for this, the first being that if the organic solution is added too rapidly, zones of high organic solvent concentration will be formed and in these zones of high concentration the enzyme material might be inactivated to some extent. The second reason for adding the organic solvent slowly is that temperature control is very important and if the organic solvent is added too rapidly it is difficult to maintain the resulting mixture within the correct temperature limits. As the concentration of the organic solvent increases, the addition rate of the remaining organic solvent may be gradually increased. As a general rule, an advantageous addition rate will be found to be a volume of organic solvent per
hour equal to from 2% to 8% of the volume of the aqueous solution of enzymes. Toward the end of the addition, the addition rate may advantageously be increased to a volume of organic solvent per hour equal to 8% to 14% of the volume of the aqueous solution.

The removal of the precipitate resulting from the addition of the organic solution may be performed by any of the well known methods such as centrifugation or filtration. However, as one is usually working with quite large volumes, a diminution of such methods may be desirable. For instance a satisfactory procedure when working with large volumes has been found to be as follows: allow the mixture to settle for from 10 to 24 hours, remove and discard the clear supernatant, and centrifuge the remaining material to obtain the precipitate. Of course, if one is working with small volumes other procedures such as simple centrifugation are usually more satisfactory.

If desired, one may employ the organic solvent in this process to obtain a fractional precipitation of the protein material in the enzyme mixture. This is as per the process of Cohen (U. S. Patent No. 2,350,074). In other words by carefully adjusting the five variables familiar to those skilled in the art and consisting of: (1) ionic strength, (2) protein concentration, (3) temperature, (4) hydrogen ion concentration, and (5) organic solvent concentration, one can obtain an additional purification of the enzymes. By this procedure, the above variables are adjusted so that the enzyme material is more readily precipitated by the organic solvent than is the other protein material in the mixture. For this reason the preferred quantity of organic solvent to be employed in the process of this invention is usually the minimum amount required to precipitate the enzymes under conditions wherein the above variables are most conducive to the precipitation of the desired enzyme in preference to the other protein material in the solution. As a general rule, such an amount of organic solvent is a volume sufficient to give a resulting solution having a concentration of from about 5% to 60% organic solvent.

As indicated above, the hydrogen ion concentration and ionic strength may be important in certain conditions in which one is attempting to obtain fractional precipitation of a protein mixture along with the hemoglobin removal, ordinarily both may be varied within wide limits with satisfactory results. In fact, for a simple hemoglobin removal, the hydrogen ion concentration and ionic strength may be varied within such wide limits that their determination is usually unnecessary. Of course if the particular enzymes to be separated are unstable at strongly alkaline or strongly acidic hydrogen ion concentrations, the process of this invention should be conducted at a pH at which the enzymes are not decomposed. Likewise the protein concentration may vary widely for a simple hemoglobin removal, although the aqueous solution should not be so dilute that difficulty will be encountered in removing the precipitate of proteinaceous material. More concentrated solutions are also advantageous for the reason that less organic solvent is required. Satisfactory results can usually be obtained with protein concentrations of from about 0.8 to 2.0 mg. of total nitrogen per cc., with the preferred range being from about .01 to 2.0 mg. of total nitrogen per cc.

During the process of this invention the temperature must be carefully controlled since the enzymes are inactivated at even moderately high temperatures when in the presence of organic solvents. After the addition of the organic solvent has been started, the temperature of the solution of enzymes should be maintained at a temperature between the freezing point of the solution of enzymes and about 5° C. An advantageous procedure comprises lowering the temperature so that it is below 0° C. after the concentration of the organic solvent in the solution of enzymes reaches about 40% by volume. In other words, as organic solvent is added, the temperature of the resulting mixture should be lowered as the concentration of organic solvent in the enzyme mixture increases. In most instances this can conveniently be accomplished by cooling coils placed within the enzyme mixture. If one wishes to make certain that there is substantially no inactivation of the enzymes, the temperature of the mixture after the addition of organic solvent has been started can be maintained at a temperature at all times within 5° C. of the freezing point of the mixture. However, such a procedure results in the formation of extremely low temperatures where high organic solvent concentrations are employed and the disadvantage of employing such low temperatures usually outweighs the disadvantage of a slight amount of enzyme inactivation. Therefore, the preferred temperature range is usually a temperature above the freezing point of the mixture between about 2° C. and --5° C. until the concentration of the organic solvent in the enzyme mixture reaches about 40% by volume and between about --5° C. and --20° C. after the concentration of organic solvent in the enzyme mixture reaches about 40% by volume. Even if only a small percentage of organic solvent is employed the temperature should be carefully watched as the addition of the organic solvent generally results in an increase in temperature of the aqueous solution of enzymes.

Ordinarily most of the hemoglobin will be present in the enzyme mixture in its reduced form. This is because a sulfhydryl reducing agent is usually present in the fermentation medium in which the enzymes are produced and this reducing agent maintains most of the hemoglobin in a reduced state. However, since cholesterol is more effective in removing the hemoglobin-O when it is in the reduced form, it is sometimes advantageous to add a sulfhydryl reducing agent to the enzyme mixture prior to cholesterol treatment thus eliminating the possibility of any appreciable quantity of the hemoglobin being in the oxidized form.

The concentration of the reconstituted solution is largely a matter of discretion as long as enough aqueous solvent is employed to dissolve the enzyme material. Of course if one employs too large an excess of aqueous solvent in preparing this reconstituted solution, difficulty may be encountered in removing the insoluble hemoglobin-cholesterol complex and therefore a relatively concentrated reconstituted solution is usually desirable. If the precipitant contains large quantities of proteinaceous materials other than the enzymes, sufficient aqueous solvent should be employed to also dissolve these impurities as otherwise appreciable loss of enzyme material might be encountered. As a general rule, a quantity of aqueous solvent sufficient to give a total nitrogen concentration in the reconstituted solution of from 0.005 mg. of total nitrogen per cc. of solution
to about 3.0 mgs. of total nitrogen per cc. of solution will be found to be advantageous.

The removal of the insoluble cholesterol and cholesterol-hemolysin complex from the reconstituted solution may be performed by any of the known methods for separating such solids from liquids, such as filtration, decantation, or centrifugation. If difficulty is encountered, aids to such processes, such as filter aids, may be employed although care must be exercised that the enzyme material is not thereby removed from the solution. Such procedures are well known to those skilled in the art and as they do not constitute an essential part of this invention will not be described in greater detail in this specification.

This invention will be illustrated by procedures for removing the hemolysin from a mixture of enzymes comprising streptokinase and streptodornase, although it should be remembered that such procedures may be employed in the removal of hemolysin-O from practically any mixture of labile enzymes.

When bacteria of the Lanasfield group A, "human C" and C are grown on a fermentation medium such as one disclosed in copending application Ser. No. 239,666 filed June 8, 1951, they produce streptolysin-O (hemolysin-O) in amounts which may vary from 10 to 1,000 Bernheimer units of streptolysin per each 100 units of streptokinase. The amount of streptolysin produced is dependent upon a number of factors, some of which are undefined, but, the streptolysin content can easily be determined by assay. This streptolysin must be removed before the enzymes can be employed in medicinal preparations with complete safety.

The streptolysin-O can be readily removed from mixtures of enzymes comprising streptokinase and streptodornase by the new process of this invention. In removing streptolysin from streptokinase and streptodornase the hydrogen ion concentration of the solution containing the enzymes should be maintained at a pH of about 2.0 to 3.5 since streptokinase and streptodornase tend to be permanently inactivated at pH's below 2.0 and at pH's above 3.5. The process may be applied to mixtures comprising streptokinase and streptodornase at widely varying ionic strengths. In fact, a solution having an ionic strength of practically 0 up to 2.0 or even higher may be satisfactorily employed so that this variable need not ordinarily be controlled. The protein concentration in the mixture comprising streptokinase and streptodornase may also vary widely although if the solution is too dilute, for instance less than 0.005 mg. of total nitrogen per cc. of solution, the precipitate will be so fine as to be difficult to recover and if the solution is too concentrated, for instance above about 3.0 mg. of total nitrogen per cc. of solution, incomplete removal of the hemolysin may be experienced. As a general rule protein concentrations between about 0.01 and 2.0 mg. of total nitrogen per cc. of solution will be found to be most satisfactory. Under these conditions a volume of organic solvent sufficient to give a resulting mixture having a concentration of organic solvent of about 5% to 60% by volume will be required for precipitation of the enzymes.

As mentioned above, the procedure of this invention may be employed under conditions wherein a fractional precipitate of the protein material is obtained if certain variables are controlled. If one wishes to obtain such a fractional precipitation of the protein material in removing streptolysin from mixtures of enzymes comprising streptokinase and streptodornase the optimum conditions are as follows: ionic strength 0.3 to 0.7, pH 3.5 to 6.5 and protein concentration in the aqueous solution of about 0.3 to 0.7 mg. of total nitrogen per cc. of solution. Under such conditions streptokinase and streptodornase are substantially completely precipitated from an aqueous solution containing a mixture of the same by organic solvents when added in a volume sufficient to give a resulting solution having a concentration of about 20% to 40% organic solvent.

The procedure for removing the streptolysin from a mixture of enzymes comprising streptokinase and streptodornase is substantially the same as outlined generally above. In a quantity of organic solvent within the limits set forth in the preceding paragraphs there is dissolved the desired amount of cholesterol ranging from 0.61 mg. to 1,000 mg. of cholesterol per 10,000 Bernheimer units of streptolysin in the aqueous solution of enzymes, and the organic solution of cholesterol is then combined slowly with the aqueous solution as one disclosed in copending application Ser. No. 239,666. During this addition, care is exercised to maintain the temperature of the enzyme mixture at about 5° C. or lower. The resulting fine dispersion of cholesterol results in the formation of an insoluble streptolysin cholesterol complex and the organic solvent results in the precipitation of the enzymes. This precipitate is removed and reconstituted in water. As the enzymes are soluble in water, there remains a fine precipitate of insoluble streptolysin-cholesterol complex and free cholesterol which may be removed by centrifugation or filtration. The purified streptokinase and streptodornase can then be recovered by the usual recovery procedures such as vacuum distillation of the aqueous solvent or by precipitation of the enzymes with a protein precipitate such as a lower alcohol, or the solution of enzymes can be employed directly in other purification procedures.

As the precipitate of streptolysin-cholesterol complex is very fine, difficulty is often encountered in removing the same. A filter aid is sometimes used. The time required for removing the fine precipitate when the reconstituted solution is also contaminated with soluble proteinaceous impurities, comprises performing a protamine purification upon the reconstituted solution. Such a protamine purification procedure constitutes the subject matter of copending application Ser. No. 240,296 filed concurrently herewith, and comprises treating the solution of enzymes with 0.05 to 1.5 parts by weight of protamine per part by weight of total nitrogen in the enzyme mixture. Such a protamine precipitation results in a voluminous precipitate of the proteinaceous impurities and this precipitate appears to occlude the finely dispersed particles of free cholesterol and streptolysin-cholesterol complex thus resulting in a clear supernatant.

The following examples are for the purpose of illustration. All parts are by weight unless otherwise indicated.

Example 1

In 30 liters of hot ethanol there is dissolved 261 grams of cholesterol. This solution is then poured into 250 liters of cold ethanol and the resulting solution cooled to about 6° C.

An aqueous solution of crude enzyme material obtained from the fermentation of streptococcal
of the Lansfield group "C" is prepared so that in a volume of 360 liters there is about 1x10^6 units of streptokinase, 2,677,648 units of streptodornase.

As the enzyme material from which the solution is prepared is crude material, the solution contains substantial quantities of enzymes other than streptokinase and streptodornase and also substantial quantities of water insoluble impurities. This solution is then cooled to a temperature of about 2°C.

The above organic solution of cholesterol is then added slowly with cooling to the aqueous solution of enzyme material. The addition rate at the start is 12 liters of organic solution per hour, which rate is increased constantly to about 40 liters per hour towards the end of the addition. During this addition the aqueous mixture is cooled by means of cooling coils so that the temperature of the mixture is lowered from about 2°C at the start of the addition to about 5°C when all of the organic solution has been added.

After the addition of the organic solution is complete, the resulting mixture is allowed to stand 18 hours or until the precipitate is well settled, at the end of which time the clear supernatant is removed and added 5 molar calcium chloride solution to a 1 liter of distilled water for each 25 grams of solid material. The insoluble cholesterol-streptokinase complex is then removed by centrifugation to leave a solution of the purified enzyme material. The enzyme material is then recovered from the aqueous solution by alcoholic precipitation.

**Example II**

The following example is for the purpose of illustrating the special procedure of removing the precipitate of hemolysin-cholesterol complex from the reconstituted solution which comprises performing a protamine precipitation upon the reconstituted solution.

227 liters of a reconstituted solution of enzyme material containing insoluble cholesterol-streptokinase complex and free cholesterol is prepared as in Example I. The pH of this slurry is adjusted to 8.0 and there is then added 5 molar calcium chloride solution until the calcium chloride molarity of the slurry is about 0.5 molar. To this mixture there added 1% protamine solution until a grayish, rapidly settling, flocculent precipitate is obtained which leaves a clear supernatant. The precipitate is allowed to settle, and the supernatant containing the enzymes is then removed by centrifugation. Additional liquid is recovered from the precipitate by centrifugation.

An assay of the original solution before cholesterol treatment gave the following values:

Total streptokinase—2.677,648 units
Total streptodornase—4,644 x 10^6 units
Total streptolysin—1,009 x 10^6 units

An assay of the supernatant obtained after protamine treatment gave the following values:

Total streptokinase—1,092 x 10^6 units
Total streptodornase—2,138 x 10^6 units
Total streptolysin—1 x 10^6 units

Thus it may be seen that the procedure results in a satisfactory recovery of the enzymes while resulting in a substantial reduction in the streptolysin content.

We claim:

1. A method of purifying labile enzymes produced by the fermentation of bacteria selected from the group consisting of *Clostridium welchii*, streptococci, and staphylococci contaminated with hemolysin-O which comprises treating an aqueous solution of enzyme containing material with a quantity of water miscible organic liquid and protein precipitant at least sufficient to result in the precipitation of the enzyme material, said organic liquid having dissolved therein an amount of cholesterol at least sufficient to form a complex with substantially all of the hemolysin-O in said solution of enzyme containing material, said amount of cholesterol being equal to at least 0.01 mg. of cholesterol per 10,000 Bernheimer units of hemolysin-O, maintaining the mixture at all times during the addition of said organic liquid at a temperature which is between the freezing point of said mixture and about 5°C, removing the resulting precipitate comprising enzyme material, hemolysin-cholesterol complex and free cholesterol, reconstituting said precipitate in aqueous solution and removing the insoluble cholesterol-hemolysin complex and free cholesterol from said reconstituted solution.

2. The method of claim 1 wherein said aqueous solution of enzyme containing material is treated with from 0.2 mg. to 2.0 mg. of cholesterol for each 10,000 units of hemolysin-O contained therein.

3. The method of claim 1 wherein said enzyme containing material comprises a mixture of streptokinase and streptodornase.

4. The method of claim 1 wherein said organic liquid protein precipitant comprises a lower aliphatic alcohol.

5. The method of claim 1 wherein said organic liquid protein precipitant is ethyl alcohol.

6. The method of claim 1 wherein the temperature of the enzyme mixture is maintained at all times during the addition of said organic liquid at a temperature above the freezing point of the mixture between about 2°C and −5°C, until the concentration of organic solvent in the enzyme mixture reaches about 40% by volume and between about −5°C and −20°C. After the concentration of organic solvent in the enzyme mixture reaches about 40% by volume.

7. The method of claim 1 wherein the temperature is maintained at all times within 5°C of the freezing point of said mixture.

8. A method of purifying labile enzymes produced by the fermentation of bacteria selected from the group consisting of *Clostridium welchii*, streptococci, and staphylococci contaminated with hemolysin-O which comprises treating an aqueous solution of enzyme containing material with a quantity of water miscible organic liquid protein precipitant comprising a lower aliphatic alcohol and having dissolved therein from 0.01 mg. to 1,000 mg. of cholesterol for each 10,000 Bernheimer units of hemolysin in said aqueous solution of enzyme material, said quantity of lower aliphatic alcohol being sufficient to give a resulting solution having a concentration of from about 5% to 50% organic solvent, lowering the temperature of the mixture during the addition of said organic liquid so that said mixture is at a temperature which is between the freezing point of said mixture and about 5°C when the concentration of organic solvent in said mixture is less than about 40% by volume and at a temperature between the freezing point of said mixture and about 0°C when the concentration...
of organic solvent in said mixture is greater than about 40% by volume, whereby said cholesterol forms a cholesterol-hemolysin complex with said hemolysin and said lower aliphatic alcohol results in the precipitation of said enzyme material, removing the precipitate comprising enzyme material, cholesterol-hemolysin complex and free cholesterol, reconstituting said precipitate in aqueous solution and removing the insoluble cholesterol-hemolysin complex and free cholesterol from said reconstituted solution.

9. The method of claim 8 wherein said organic liquid protein precipitant is ethyl alcohol.

10. The method of claim 8 wherein the ionic strength is maintained at a value of from 0.3 to 0.7, the protein concentration is maintained at a level to give a total nitrogen concentration per cc. of about 0.3 to 0.7 milligram and the hydrogen ion concentration of the aqueous solution of enzyme containing material is maintained at a level to give a pH reading of 3.5 to 6.5, whereby the desired enzyme material is precipitated in preference to the other protein material in said solution when treated with said water miscible organic liquid protein precipitant.

11. The method of claim 8 wherein a sulfhydryl reducing agent is added to said aqueous solution of enzyme containing material before precipitation.

12. The method of claim 8 wherein the temperature of the mixture is lowered during the addition of said organic liquid so that said mixture is at a temperature which is above the freezing point of the mixture between about 2° C. and −5° C. until the concentration of organic solvent in the enzyme mixture reaches about 40% by volume and between about −5° C. and −30° C. after the concentration of organic solvent in the enzyme mixture reaches about 40% by volume.

13. A method of purifying enzyme material comprising a mixture of streptokinase, streptodornase and proteinaceous impurities and which is contaminated with streptolysin-O, which comprises treating an aqueous solution of the streptokinase and streptodornase containing material with a quantity of water miscible organic liquid protein precipitate comprising a lower aliphatic alcohol and having dissolved therein from 0.05 mg. to 50 mg. of cholesterol for each 10,000 Bernheimer units of streptolysin-O in said aqueous solution, said quantity of lower aliphatic alcohol being sufficient to give a resulting solution having a concentration of from about 5% to 60% organic solvent, maintaining the mixture at all times during the addition of said organic liquid at a temperature which is between the freezing point of said mixture and about 5° C., whereby said cholesterol forms a cholesterol-streptolysin complex with said streptolysin and said lower aliphatic alcohol results in the precipitation of said enzyme material, removing the precipitate comprising enzyme material, cholesterol-streptolysin complex and free cholesterol, reconstituting said precipitate in aqueous solution, treating said reconstituted solution with 0.05 to 1.0 parts by weight of protamine per part by weight of total nitrogen in said reconstituted solution, and removing the resulting precipitate comprising impurities precipitated by said protamine, cholesterol-streptolysin complex and free cholesterol.

14. The method of claim 13 wherein said organic liquid protein precipitant is ethyl alcohol.

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