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ACTIVATED ENZYME COMPOSITIONS

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This invention relates to the activation of proteolytic enzymes.

In recent years a considerable amount of research effort has been devoted to the study of a class of biological catalysts commonly known as enzymes. Notable advances have been made in this field and vast quantities of scientific data have been accumulated regarding physiological and chemical properties of enzymes. A number of them have been prepared in highly purified form and their chemical constitution has been studied in detail. It has been established, for example, that enzymes are largely protein in nature; that their essential structural unit is a long chain arising from the condensation of a number of amino acid residues joined through peptide linkage; that they are large size molecules of high molecular weight; and that, in solution, they migrate characteristically in an electrical field.

Because enzymes are not as yet structurally identifiable, their characteristics are usually established along functional lines. Thus, it is typical of some enzymes that they are, under optimal conditions of temperature, either inhibited or not affected at all when chemical agents are added to them. Others demonstrate an accelerated activity in the presence of chemicals. In fact, some enzymes are completely inactive in the absence of chemical additives. Agents which are capable of increasing the activity of such weakly active or inactive enzymes are called activators.

The search for suitable activators has been considerably intensified in recent years due to the discovery that proteolytic enzymes are useful in the medical field in the physiological debridement of eschar, blood and pus. Although a large number of proteolytic enzymes are available, laboratory tests show that their proteolytic activity is weak and slow in onset, thus reducing their applicability from an industrial point of view.

It has now been found that certain proteolytic enzymes are activated in the presence of ions of calcium, magnesium, manganese, cobalt or combinations thereof. Preferred for use as single activators are calcium and magnesium. Where a combination of two metals is employed to activate the enzyme, calcium and magnesium are preferred. The proportions of the metal ions to be used in activating proteolytic enzymes according to this invention may be varied considerably, depending upon the number of different activating ions employed, the pH of the final product desired and other factors. Satisfactory results are obtained when the ions are added in a non-toxic concentration from about 0.01 molar to about 1.0 molar. For optimal activation, it is preferred to use a concentration from about 0.05 molar to about 0.1 molar.

Useful non-toxic salts of the metal ion activators include chlorides, acetates, lactates, propionates, gluconates and, preferably, phosphates. The addition of alkali

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metal ions, i.e. potassium and sodium, preferably in the form of phosphates or acetates, is advantageous primarily for the purpose of buffering the enzyme compositions. For topical use on the animal organism it has been found that adjustment of pH from about 3.0 to about 5.0, optimally from about 3.0 to about 4.0 is satisfactory. Further, it is also desirable to include a reducing agent such as a thiosulfate, i.e. sodium or potassium or, preferably, cysteine in order to obtain maximum results. The inclusion of cysteine in a concentration from about 0.05 molar to about 0.1 molar is satisfactory.

The term "proteolytic enzyme" as used herein is meant to include pinguinain, papain, ficin, bromelain, bromelain and asclepain. Preferred for use as the proteolytic enzyme is pinguinain or ficin; especially preferred is pinguinain.

"Pinguinain" as used in this invention is meant to include the proteolytic enzyme described by Asenjo (Science, v. 95, No. 2454, pp. 48-49) as well as the improved form of pinguinain obtained by macerating the fruit of *Bromelia pinguin*, separating the juice from the fibrous debris, spray-drying and, if desired, further purifying by precipitation with ammonium sulfate, aqueous methanol, aqueous ethanol or aqueous isopropanol. Pinguinain is a high molecular weight enzyme system comprised essentially of three components, two of which (A and B) are proteinaceous in character and readily characterizable by their mobilities in an electrophoretic field, the third (C) being non-protein and lacking in electrophoretic mobility between pH 3.5 and pH 6.0. The average electrophoretic mobility in 0.1 ionic glycine buffer at pH 3.5 for A component is 0.377, for B component it is 0.234. The average mobility in acetate buffer at pH 4.6 for A component is 0.175, for B component it is 0.064. The isoelectric point for A component is above pH 6.0, for B component pH 5.25. The enzyme demonstrates optimal proteolytic activity (in an Azocoll substrate) at a pH from about 5.2 to about 5.5 and at pH 7.3, and is inactivated at temperatures in excess of 80° C. It is not activated by ascorbic acid, but is activated by sodium thiosulfate and cysteine. It is soluble in water, normal saline and aqueous salt solutions of low ionic strength.

In actual practice, the activated enzymes of this invention may be used in the form of aqueous solutions either for topical administration or for intramuscular injection. They may also be used in the form of gels suitable for application on burned or ulcerated areas. The quantity of activated enzyme to be employed in such formulations will depend largely upon the needs of the operator, the condition of the animal organism and the nature of the malady being treated. For example, a formulation containing activated pinguinain intended for topical application, i.e. an aqueous irrigation solution or a gel, should contain from about 1% to about 15%, preferably about 10% enzyme on a weight basis, or from about 1,000 to about 30,000 Azocoll units per gram or milliliter. For purposes of injection, an appropriately buffered aqueous solution of activated pinguinain should contain from about 0.01% to about 1%, preferably about 0.5% enzyme on a weight basis, or from about 1,000 to about

20,000 Azocoll units per milliliter. Similar proportions may be employed when any one of the other enzymes specified hereinabove is formulated. To ensure maximum proteolytic activity, activated enzyme solutions and gels for topical use should be adjusted to a pH from about 3.0 to about 5.0 with an appropriate buffer, preferably acetate or phosphate. Solutions intended for intramuscular injection should be adjusted to near neutrality. The utility of this material for use in human therapy has not as yet been clinically established.

Among the substances suitable for formulation of activated enzyme gels there may be mentioned vegetable gums such as acacia, tragacanth, ghatti or karaya; mucilages such as those obtained from guar, bean, linseed, the locust bean or other related leguminous plant seeds; or cellulose derivatives such as carboxymethylcellulose, methylcellulose, starch or starch derivatives. It is to be understood that although the activated enzyme compositions of this invention may find primary application as debriding and anti-inflammatory agents, they are also useful in a wide variety of industrial processes where proteolytic enzymes are conventionally employed, as for example in chill-proofing of beer, cheese-making, leather manufacture, as meat tenderizers, etc., and it is intended that these applications be included within the scope of this invention.

The Azocoll unit as used herein is an index of proteolytic activity as determined by the method of Bidwell (Biochemical Journal, 46, pp. 589-598, 1950) and Oakley et al. (Journal of Pathology and Bacteriology, 58, pp. 229-235, 1946).

Enhancement of proteolytic activity in the presence of metal ions is demonstrable by means of tests on guinea pig eschar. The procedure is as follows: The hair is removed from a guinea pig back with an animal hair clipper. The animal is then injected with sodium pentobarbital. The animal's back is then immersed in a water bath at 70° C. for 45 seconds. A burned area of about 2 to 3 inches in diameter is formed as a result of this immersion. Seven days after the animal has been burned, it is sacrificed and the eschar is harvested. The eschar is removed from the animal with a scalpel and stored in the frozen state until needed for use. Immediately before use, while it is still in the frozen state, the eschar is cut into pieces 1 x 1 cm. for a 5 ml. volume of reaction mixture; 1 x 2 cm. for a 10 ml. volume of reaction mixture, and 1 x 3 cm. for a 15 ml. volume of reaction mixture. The eschar pieces are permitted to thaw about 10 minutes before they are placed in the reaction mixtures. The reaction mixture containing a piece of eschar is incubated in an oven at 37° C. for between 16 and 18 hours. The eschar is then inspected visually and the activity of the preparation is evaluated. The term "reaction mixture" as used in these tests means the mixture comprising the eschar substrate, the enzyme, and the particular carrier employed.

Following are the results of in vitro tests on guinea pig eschar using pinguinain:

Enzyme conc., percent	Azocoll value units/ml.	Buffer	pH	Conc. of cysteine (M)	Metal-ion	Result
6	285×10 ²	Water	3.4	0.008m	None	2
6	285×10 ²	do	3.4	0.008m	0.2M cobalt chloride	4
6	285×10 ²	do	3.4	0.008m	0.2M manganese chloride	3
6	285×10 ²	do	3.4	0.008m	0.02M magnesium chloride	5
6	285×10 ²	do	3.4	0.008m	0.2M cobalt chloride	6
6	285×10 ²	do	3.4	0.008m	0.2M manganese chloride	7
6	285×10 ²	do	3.4	0.008m	0.2M magnesium chloride	6
6	285×10 ²	do	3.4	0.008m	0.2M calcium chloride	7
4	182×10 ²	do	4.3	0.05 m	0.02M calcium chloride, 0.04M magnesium chloride	6
10	270×10 ²	Water-HCl	3.0	0.008m	do	7
10	270×10 ²	Water	4.0	0.008m	do	5
10	270×10 ²	Water-NaOH	5.2	0.008m	do	5
10	270×10 ²	do	9.8	0.008m	do	2

Key to Evaluation of In Vitro Tests on Guinea Pig Eschar

Value	Description
5 0-----	No activity.
1-2-----	Separation of layers with little or no digestion.
3-----	Separation of layers and some digestion.
4-----	Fair digestion with few small pieces broken off the main body of eschar.
5-----	Good digestion with the eschar partially disintegrated.
6-----	Eschar completely disintegrated with a few small particles remaining.
10 7-----	Eschar completely disintegrated.

The activating effect of metal ions on proteolytic enzymes is also evidenced by in vivo tests on guinea pig eschar. The procedure is as follows: Hair is removed from a guinea pig back with an animal hair clipper. The animal is then injected with sodium pentobarbital. The animal's back is then immersed in a water bath at 70° C. for 45 seconds. A burned area of about two to three inches in diameter is formed as a result of this immersion. Seven days after the burn, the animal is treated with the enzyme preparation, applied in ointment form to the burned area on three successive days. The ointment is covered with a film which is kept in place by tape. Observations of the percentage of eschar which was debrided on each animal tested are recorded each day that the bandages are removed. The bandage is removed and the eschar is observed on the first, second and third days after the initial application of enzyme. The ointment employed in these tests consisted of 3 g. of guar gum, 1.8 g. of pinguinain and 30 ml. of water. The enzyme and gum were mixed in a dry state and added to the water immediately before use.

Results of in vivo tests on guinea pig eschar employing pinguinain are given below. The figure "percent debridement" represents the percentage of the eschar which was debrided on the animal tested. Complete cleaning of the wound to the viable graft tissue is considered 100%.

Amount of enzyme applied per animal in total treatment (azocoll units)	Diluent	Percent debridement		
		24 hrs.	48 hrs.	72 hrs.
220×10 ³	Acetate	---	---	50
710×10 ³	do	---	---	60
420×10 ³	Water	5	70	85
381×10 ³	0.5M calcium chloride acetate buffer.	5	90	80
381×10 ³	0.1M calcium chloride phosphate buffer.	5	95	95
381×10 ³	0.1M magnesium chloride phosphate buffer.	5	95	97
381×10 ³	0.1M calcium chloride, 0.1M magnesium acid phosphate phosphate buffer.	88	95	98
381×10 ³	0.05M calcium chloride, 0.05M magnesium acid phosphate phosphate buffer.	10	96	100
381×10 ³	0.05M calcium chloride, 0.05M magnesium acid phosphate phosphate buffer.	60	98	100
381×10 ³	0.05M calcium chloride, 0.05M magnesium acid phosphate phosphate buffer.	75	95	100
381×10 ³	0.05M calcium chloride, 0.05M magnesium acid phosphate phosphate buffer.	25	99	100
381×10 ³	0.05M calcium chloride, 0.05M magnesium acid phosphate phosphate buffer.	85	98	100
381×10 ³	0.05M calcium chloride, 0.05M magnesium acid phosphate phosphate buffer.	75	95	100
381×10 ³	0.05M calcium chloride, 0.05M magnesium acid phosphate phosphate buffer.	50	95	100

The following tests illustrate the activating ability of metal ions on other proteolytic enzymes. The tests were conducted as follows: A 1 x 1 cm. piece of eschar was placed in each enzyme solution (5 ml.). The enzyme solution was prepared by weighing enzyme quantities and cysteine and adding 20 ml. of diluent activator, or buffer without activator as the case may be, to the weighed quantities of enzyme. One piece of eschar was added to 5 ml. of enzyme solution in a test tube. The reaction mixture was incubated in an oven at 37° C. for between 16 and 18 hours and the results of the test evaluated. The diluent activator used in these tests (and which may be employed in actual practice for the purposes specified hereinabove) had the following composition:

Calcium chloride	G.
Magnesium acid phosphate	0.55
Disodium phosphate	1.09
Distilled water, q.s.	0.057
100.00 ml.	

The results are as follows:

Enzyme	Diluent	Cysteine, mg.	pH	Results
2g. papain	0.1M sodium acetate pH 3.75.	120	4.3	Eschar partially disintegrated.
Do.....	Diluent activator	120	4.3	Eschar completely disintegrated.
2g. ficin	0.1M sodium acetate pH 3.75.	120	4.4	Eschar partially disintegrated.
Do.....	Diluent activator	120	4.4	Eschar completely disintegrated.

What is claimed is:

1. An activated enzyme composition adjusted to a pH of from about 3.0 to about 5.0 comprising a plant proteolytic enzyme selected from the group consisting of pinguinain, papain, ficin, bromelin, bromelain and asclepain and, in non-toxic concentration, at least one metal ion selected from the group consisting of calcium, magnesium, manganese and cobalt in the form of their non-toxic salts.

2. A composition as set forth in claim 1 wherein the metal ions are contained in a concentration from about 0.01 molar to about 1.0 molar.

3. A composition as set forth in claim 1 wherein the metal ions are contained in a concentration from about 0.05 molar to about 0.1 molar.

4. An activated enzyme composition adjusted to a pH of from about 3.0 to about 5.0 comprising a plant proteolytic enzyme selected from the group consisting of pinguinain, papain, ficin, bromelin, bromelain and asclepain, at least one metal ion selected from the group consisting of calcium, magnesium, manganese and cobalt in the form of their non-toxic salts and at non-toxic concentrations, and a buffer.

5. A composition as set forth in claim 4 wherein the buffer is an acetate.

6. A composition as set forth in claim 4 wherein the buffer is a phosphate.

7. An activated enzyme composition adjusted to a pH of from about 3.0 to about 5.0 comprising a plant proteolytic enzyme selected from the group consisting of pinguinain, papain, ficin, bromelin, bromelain and asclepain, at least one metal ion selected from the group consisting of calcium, magnesium, manganese and cobalt in the form of their non-toxic salts and at non-toxic concentrations, a buffer and cysteine.

8. An activated enzyme composition adjusted to a pH of from about 3.0 to about 5.0 comprising a plant proteolytic enzyme selected from the group consisting of pinguinain, papain, ficin, bromelin, bromelain and asclepain, at least one metal ion selected from the group consisting of calcium, magnesium, manganese and cobalt in the form of their non-toxic salts and at

non-toxic concentrations, a buffer and an alkali metal thiosulfate.

9. An activated enzyme composition adjusted to a pH of from about 3.0 to about 5.0 comprising a plant proteolytic enzyme selected from the group consisting of pinguinain, papain, ficin, bromelin, bromelain and asclepain, at least one metal ion selected from the group consisting of calcium, magnesium, manganese and cobalt in the form of their non-toxic salts and at non-toxic concentrations, a buffer, cysteine and a pharmaceutical carrier.

10. A composition as set forth in claim 9 wherein the pharmaceutical carrier is an aqueous carrier.

11. A composition as set forth in claim 9 wherein the pharmaceutical carrier is a gel.

12. An activated enzyme composition adjusted to a pH of from about 3.0 to about 5.0 comprising pinguinain and, in non-toxic concentration at least one metal ion selected from the group consisting of calcium, magnesium, manganese and cobalt in the form of their non-toxic salts.

13. An activated enzyme composition adjusted to a pH of from about 3.0 to about 5.0 comprising pinguinain, at least one metal ion selected from the group consisting of calcium, magnesium, manganese and cobalt in the form of their non-toxic salts and at non-toxic concentrations and a buffer.

14. An activated enzyme composition adjusted to a pH of from about 3.0 to about 5.0 comprising pinguinain, at least one metal ion selected from the group consisting of calcium, magnesium, manganese and cobalt in the form of their non-toxic salts and at non-toxic concentrations, a buffer and cysteine.

15. An activated enzyme composition adjusted to a pH of from about 3.0 to about 5.0 comprising pinguinain, at least one metal ion selected from the group consisting of calcium, magnesium, manganese and cobalt in the form of their non-toxic salts, a buffer and an alkaline metal thiosulfate.

16. An activated enzyme composition adjusted to a pH of from about 3.0 to about 5.0 comprising pinguinain, at least one metal ion selected from the group consisting of calcium, magnesium, manganese and cobalt in the form of their non-toxic salts and at non-toxic concentrations, a buffer, cysteine and a pharmaceutical carrier.

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