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(54) TOXOID DERIVED FROM THE PROTEASE OF PSEUDOMONAS AERUGINOSA, AND THE PRODUCTION THEREOF

(71) We, SHIONOGI & CO., LTD., a Japanese Body Corporate, of 12, 3-chome, Doshomachi, Higashi ku, Osaka, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a toxoid derived from the protease of Pseudomonas aeruginosa and to the production thereof. Furthermore, the invention is also concerned with the prevention and treatment of infections caused by P. aeruginosa by inoculation of this toxoid.

Infections by P. aeruginosa have presented a serious problem in the medical field. The resulting infectious diseases are observed in immature infants, newborn infants or in leukemia and cancer patients during the terminal stage. Patients suffering with such infections are debilitated in their defence mechanism and easily pass into a dangerous condition. In the field of veterinary medicine, hemorrhagic pneumonia in minks caused by P. aeruginosa and mammitis in bovines caused by P. aeruginosa give rise to a serious economic problem in the livestock industry.

Only a few antibiotics for use against P. aeruginosa have been developed recently and they are of insufficient effect. Meanwhile, the protease and elastase produced from some species of P. aeruginosa have been found to be amongst the pathogens of infections caused by P. aeruginosa as exemplified above (K. Kawaharajo et al., Japan J. Exp. Med., *45*, 79—88 (1975); Japan J. Exp. Med., *45*, 515 (1975); Japan J. Exp. Med., *44*, 435—442 (1974); J. Y. Homma et al., Japan J. Exp. Med., *45*, 89—100 (1975)).

Attempts have been made to prepare toxoids derived from the protease and elastase of P. aeruginosa.

According to the present invention there is provided a toxoid derived from the protease of Pseudomonas aeruginosa, which toxoid is obtainable by inactivating the protease and has the following physicochemical properties:

(1) Molecular weight: 63000 (gel filtration)
(2) Ultraviolet absorption spectrum: maximum 280 mμ ($E^{280}_{1\%}$ 9.27, 0.1 M KCl), minimum 250 mμ
(3) Isoelectric point: pH 5.2 (forcal electrophoresis)
(4) Constituent amino acids: (amino acid (g)/100 g of protein) aspartic acid (15.6), glutamic acid (9.5), leucine (8.7), alanine (8.5), glycine (7.7), serine (7.6), tyrosine (6.9), phenylalanine (5.9), threonine (5.0), valine (5.0), lysine (4.1), isoleucine (3.9), arginine (2.3), tryptophan (2.3), proline (2.1), histidine (1.9), ammonia (1.4), (total 98.5 g)
(5) Appearance: colorless powder
(6) Antigen activity: positive
(7) Protease activity: negative

The toxoid having the above properties is a novel substance and is useful in medical and veterinary fields.

The prevent invention has been divided out of our co-pending Application No. 3899/77 (Serial No. 1 560,861) which describes and claims a toxoid derived from the elastate of Pseudomonas aeruginosa, which toxoid is obtainable by inactivating the elastase and has the following physicochemical properties:

(1) Molecular weight: 47000 (gel filtration)
(2) Ultraviolet absorption spectrum: maximum 278mμ ($E^{278}_{1\%}$ 21.2, 0.1 M KCl), minimum 252 mμ
(3) Isoelectric point: pH 6.5 (electrophoresis with cellulose acetate membrane
(4) Constituent amino acids: (amino acid (g)/100 g of protein) aspartic acid (14.2), tyrosine (9.9), phenylalanine (7.0), glutamic acid (6.5), arginine (6.5), alanine (5.8), glycine (5.6), serine (5.6), threonine (5.0), valine (4.9), leucine (4.3), lysine (3.9), methionine (2.9), proline (2.9), isoleucine (2.7), histidine (2.6), tryptophan (2.3), cystine/2 (1.2), ammonia (0.9), (total 94.7 g)
(5) Appearance: colorless powder
(6) Antigen activity: positive
(7) Elastase activity: negative

The toxoid provided by the present invention and the toxoid provided by the invention described and claimed in our co-pending Application No. 3899/77 (Serial No. 1 560 861) are effective in suppressing the infections caused by P. aeruginosa when used individually or in combination.

Reference is herein made also to co-pending Application No. 3906/77 (Serial No. 1 546 035), of which we are joint Applicants, which describes and claims a mixed vaccine against infections caused by Pseudomonas aeruginosa which comprises as the antigens an elastase toxoid obtained from P. aeruginosa and a protease toxoid obtained from P. aeruginosa.

The toxoid of the present invention may be manufactured in the following manner:— The protease from P. aeruginosa is dissolved in a suitable buffer of pH 6 to 10, e.g. a borate buffer, an acetate buffer, a phosphate buffer, a glycine buffer, or a tris buffer. A buffer having pH 9 is preferred. To the enzyme solution in buffer is added formalin or oxymethanesulfinic acid until the enzyme is inactivated. Formalin is advantageously used at a concentration of from 4 to 10% v/v) and oxymethanesulfinic acid of from 0.4 to 3 M concentration in the presence of e.g. lysine. Arginine, leucine, tyrosine, glutamic acid, and the like can be used instead of lysine, but lysine is the most preferred. The preparation is preferably affected at from 10 to 40°C, more preferably at room temperature. The reaction period depends upon the other reaction conditions, being from 1 to 7 days under favourable conditions, preferably 3 days or more for inactivating the enzyme.

The starting materials, the protease from P. aeruginosa, may be obtained by a known method (Japanese Patent Publication No. 27315/1965). The properties of the protease are also described in the aforementioned Japanese Patent Specification.

The efficacy of the thus-obtained toxoid (abbreviated as "protease toxoid") is demonstrated by immune tests on rabbits, mice and minks.

Experiment 1 Antigenicity Test on Rabbits

(1) Method:

Protease toxoid (1 mg) is subcutaneously inoculated into three New Zealand white rabbits with Freund's incomplete adjuvant. After 1 week, protease toxoid (1 mg) is again subcutaneously injected followed by intramuscular inoculations of protease toxoid (1 mg) at intervals of two weeks, two weeks, three weeks and one week, successively. Blood is collected from an ear vein at certain time intervals. Blood serum is separated and heated to 56°C to measure the passive hemagglutinative (PHA) titer by the method described in Japan. J. Exp. Med. Vol. *45*, No. 5, 361—365 (1975), i.e. the passive hemagglutination reaction using the protease as an antigen.

Inoculation No.	1st	2nd	3rd	4th	5th
Protease Toxoid	1 mg*	1 mg*	1 mg	1 mg	1 mg
Administration Route**	s.c.	s.c.	i.m.	i.m.	i.m.
(Interval)	↓ (2 weeks)	↓ (2 weeks)	↓ (2 weeks)	↓	(3 weeks) ↓ (1 week)
PHA Titer	↑	↑	↑	↑	↑ ↑
Rabbit No. 1	0	512	1024	1024	1024 1024
No. 2	0	128	256	256	256 256
No. 3	0	128	512	512	512 4096

Note:
* Inoculation with Freund's incomplete adjuvant
** s.c. = subcutaneous injection
i.m. = intramuscular injection

Experiment 2 Antigenicity Test on Minks

Thirteen Sapphire minks are intramuscularly inoculated with protease toxoid (1000 γ) with adjuvant (potassium alum). The second vaccination is performed with the toxoid (500 γ) about 2 weeks later. The PHA titer against protease increased from 64 to 512 (reciprocal serum of dilution), though PHA titer is not observed before the vaccination.

Experiment 3 Enzyme Neutralizing Activity of Rabbit Serum Vaccinated with Toxoids

(1) Method:

Protease-neutralizing activity is examined by measuring protease activity. A rabbit serum vaccinated with protease toxoid and a normal rabbit serum are tested. The PHA titer of the vaccinated serum is 7860. The PHA titer of the normal serum is negative against protease, elastase and OEP. The sera are heated at 56°C for 30 minutes. A certain desired amount of protease is dissolved in 1/15 M phosphate buffer (pH 7.4). To the protease solution (0.2 ml) is added the serum to be tested (0.2 ml) and then physiological saline to make the total volume up to 3.0 ml. The mixture is kept at 37°C for 60 minutes. The remaining protease activity is measured by the method described below.

To the solution obtained above in a desired concentration is added a 2% (v/v) casein solution (1 ml). The mixture is incubated at 40°C for 10 minutes followed by addition of a solution (2 ml) containing 0.1 M trichloroacetic acid, 0.2 M acetic acid and 0.2 M sodium acetate to stop enzyme activity. The mixture is kept at the same temperature for 2 hours to precipitate unreacted casein completely and is then filtered. The quantity of tyrosine is measured in the filtrate by the Foline method. In this method, a 0.4 M solution (5 ml) of sodium carbonate and a 20% (v/v) solution of Phenol Reagent Solution (produced by Nakai Chemical Co., Kyoto, the acid concentration being 1.8 N) are added to the filtrate (1 ml). After 15 to 20 minutes, the absorbance of the mixture is measured at 670 mμ. The quantity of free tyrosine is

calculated by comparing with the value obtained from unincubated toxoid-casein mixture.

(2) Results:

Protease γ/0.2 ml	Remaining Protease Activity (Neutralizing Activity) (OD 670 mμ)		
	Vaccinated Serum*	Normal Serum	Without Serum
0	0.058 (7860)	0.07	0.045
10	0.062 (1920)	0.15	0.105
20	0.060 (240)	0.21	0.17
40	0.25 (<120)	0.38	0.30
80	0.46 (<120)	0.55	0.46
160	0.75 (<120)	0.70	0.76

Note: * Figures in the parenthesis indicate PHA titer.

As shown in the above experiments, inoculation of the toxoid of the present invention results in a PHA titer and the production of neutralizing antibodies. It is, therefore, effective against infections caused by P. aeruginosa.

The toxicity of the toxoid derived from the protease is very weak. Intraperitoneal administration (i.p.) to mice at a dose of 1 mg/mouse does not reveal any acute toxicity, the minimum lethal dose being 0.2 mg/mouse (i.p.).

The toxoid of this invention can be applied to human beings and other animals to prevent and treat infections by P. aeruginosa. The inoculation may be practised subcutaneously, intramuscularly, or intracutaneously, if desired, with an adjuvant. The toxoid may also be used to produce anti-serum against the protease of P. aeruginosa. Antibodies may be collected from the anti-serum. The thus-obtained anti-serum and antibodies can be used to prevent and treat infections caused by P. aeruginosa.

Vaccines containing the toxoid can be manufactured by conventional procedures for preparing vaccines for human beings and other animals. Thus, the toxoid may be dissolved in a suitable solvent, if desired, with an adjuvant. Antiseptics may also be used, if necessary. Suitable solvents are distilled water, physiological saline and phosphate-buffered aqueous sodium chloride solution. Examples of suitable adjuvants are aluminium hydroxide, aluminium phosphate, calcium phosphate, alum and Freund's incomplete adjuvant. The amount of the adjuvant may be appropriately selected from the range of amounts which is necessary and sufficient for increasing immunoactivity. Thimerosal, phenol, benzoic acid and formalin are suitable antiseptics.

When immunization is carried out 2 to 3 times per week (without adjuvant), 1 to 50 γ/kg of protease toxoid is administered to human beings. Depending upon the state of immunization, the amounts may be increased appropriately.

The invention will now be further described and illustrated by way of the following Examples.

Example 1.

Crystalline protease of P. aeruginosa (100 mg) is dissolved in 0.05 M solution of sodium hydrogen phosphate (pH 8.5) (100—200 ml) containing 0.005—0.2 M lysine. To the solution is added formalin until the concentration reaches about 1—8% (v/v). After being allowed to stand at room temperature for 3 days, the mixture is dialyzed against water and lyophylized to yield a toxoid of the protease. Yield is 98—100%.

The remaining protease activity is examined by the method described in Experiment 3 (see earlier) after dialysis for 3 days. The results are shown in Tables 5 and 6.

TABLE 5

Concentration of Formalin	Remaining Protease Activity (%)
8	21
4	42
1	93
0	100

Note: The concentrations of toxoid and lysine are 1 mg/ml and 0.05 M, respectively.

TABLE 6

Concentration of Lysine	Remaining Protease Activity (%)
0	88
0.05	21
0.1	9
0.2	<5
Control *	100

Note: The concentrations of toxoid and formalin are 1 mg/ml and 8 %, respectively.

* no addition of formalin.

Example 2.

A toxoid is prepared in the same manner as in Example 1 using 0.5 M oxymethanesulfinic acid in place of formalin in the presence of 0.05 M lysine. The remaining protease activity is 15%.

Example 3.

A toxoid is prepared in the same manner as in Example 1 using 2.5 M oxymethanesulfinic acid in place of formalin in the presence of 0.05 M lysine. The protease activity completely disappears.

WHAT WE CLAIM IS:—

1. A toxoid derived from the protease of Pseudomonas aeruginosa, which toxoid is obtainable by inactivating the protease and has the following physicochemical properties:

(1) Molecular weight: 63000 (gel filtration)

(2) Ultraviolet absorption spectrum: maximum 280 mμ ($E^{280}_{1\%}$ 9.27 0.1 M KCl), minimum 250 mμ

(3) Isoelectric point: pH 5.2 (forcal electrophoresis)

(4) Constituent amino acids: (amino acid (g)/100 g of protein) aspartic acid (15.6), glutamic acid (9.5), leucine (8.7), alanine (8.5), glycine (7.7), serine (7.6), tyrosine (6.9), phenylalanine (5.9), threonine (5.0), valine (5.0), lysine (4.1), isoleucine (3.9), arginine (2.3), tryptophan (2.3), proline (2.1), histidine (1.9), ammonia (1.4), (total 98.5 g)

(5) Appearance: colorless powder

(6) Antigen activity: positive

(7) Protease activity: negative.

2. A process for preparing a toxoid as claimed in claim 1, which process comprises treating the protease produced from Pseudomonas aeruginosa in a buffer solution with formalin or oxymethanesulfinic acid.

3. A process as claimed in claim 2 in which formalin is used at a concentration of from 4 to 10% (v/v).

4. A process as claimed in claim 2 in which 0.4 to 3 M oxymethanesulfinic acid is used.

5. A process as claimed in any one of claims 2 to 4 which is effected at a temperature of from 10 to 40°C.

6. A process as claimed in any one of claims 2 to 5 which is effected over a period of 3 days or more.

7. A process as claimed in any one of claims 2 to 6 wherein the treatment is effected in the presence of an amino acid.

8. A process as claimed in claim 7, wherein the amino acid is lysine.

9. A process as claimed in claim 7, wherein the amino acid is arginine, leucine, tyrosine or glutanic acid.

10. A process substantially as hereinbefore described in any one of the Examples.

11. A toxoid as claimed in claim 1 which has been prepared in a process as claimed in any one of claims 2 to 10.

12. A vaccine comprising a toxoid component in a form suitable for inoculation, which component is a toxoid as claimed in claim 1 or claim 11.

13. A vaccine as claimed in claim 2 which also comprises an antiseptic and/or an adjuvant.

14. A vaccine as claimed in claim 2 and substantially as hereinbefore described.

15. A method for preventing or treating infections in a non-human animal caused by Pseudomonas aeruginosa, which method comprises inoculating the animal with a toxoid as claimed in claim 1 or claim 11, or a vaccine as claimed in any one of claims 12 to 14, in sufficient quantity to produce protease antibodies.

16. A method as claimed in claim 15 substantially as hereinbefore described in Experiment 1 or Experiment 2.

17. An anti-serum for preventing or treating infections caused by Pseudomonas aeruginosa and which is obtained from human or animal serum by inoculating a human or animal with a toxoid as claimed in claim 1 or claim 11, or a vaccine as claimed in any one of claims 12 to 14.

18. An anti-serum as claimed in claim 17 substantially as hereinbefore described in Experiment 3.

19. Pseudomonas aeruginosa protease antibodies which have been produced in a method as claimed in claim 15 or claim 16 or which have been isolated from an anti-serum as claimed in claim 17 or claim 18.

20. A method for preventing or treating infections in a non-human animal caused by Pseudomonas aeruginosa, which method comprises inoculating the animal with an anti-serum as claimed in claim 17 or claim 18 or with antibodies as claimed in claim 19.

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