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(54) **METHODES DE PREPARATION DE L'ANATOXINE
COQUELUCHEUSE**

(54) **METHOD FOR PREPARING PERTUSSIS TOXIN TOXOID**

(57) Disclosed is a method for preparing pertussis toxin toxoid by a treatment of pertussis toxin with formaldehyde, which comprises carrying out said treatment in the presence of lysine or glycine in combination with N-acetyltryptophan. The resultant toxoid does not exhibit toxicity reversion, thereby making the toxoid highly suitable for use in pertussis vaccines.



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ABSTRACT OF THE DISCLOSURE

Disclosed is a method for preparing pertussis toxin toxoid by a treatment of pertussis toxin with formaldehyde, which comprises carrying out said treatment in the presence of lysine or glycine in combination with N-acetyltryptophan. The resultant toxoid does not exhibit toxicity reversion, thereby making the toxoid highly suitable for use in pertussis vaccines.

METHOD FOR PREPARING PERTUSSIS TOXIN TOXOID

FIELD OF THE INVENTION

The present invention relates to a method for toxoidizing pertussis toxin, more particularly, to a novel method for preparing pertussis toxin toxoid from pertussis toxin by which the toxoid will not exhibit toxicity reversion, thereby making the toxoid highly suitable for use in pertussis vaccines having no substantial side effects.

PRIOR ART

Pertussis toxin is a protein produced by Bordetella pertussis and, because of the variety of its physiological and biological activities, is also referred to as leukocytosis promoting factor (LPF), histamine sensitizing factor (HSF), or islets activating protein (IAP). In addition, pertussis toxin is known to exhibit such biological activities as promotion of vascular permeability, CHO-cell clustering activity , and ADP-ribosylase activity.

It is also known that pertussis toxin is one of the most important antigens against infection with pertussis. Thus, attempts have been made to develop various pertussis vaccines in-

dispensably containing pertussis toxin toxoid by treating pertussis toxin so as to destroy its toxicity while retaining its immunogenicity.

In treating pertussis toxin for obtaining the toxoid thereof, formaldehyde and glutaraldehyde have been most commonly used. However, it has recently been pointed out that treatment with formaldehyde and/or glutaraldehyde does not sufficiently inactivate the pertussis toxin and thus does not provide the toxoid which is totally devoid of all of the toxic physiological and biological activities. For example, Sato et al. have shown that the use of formaldehyde or glutaraldehyde for producing the toxoid from the pertussis toxin only decreases to a limited extent such properties as ADP-ribosylase activity and islets activating property, while substantially decreasing such properties as leukocytosis promoting activity, histamine sensitizing activity and hemagglutination activity [Journal of Bacteriology in Japan, 42, 288(1987)]. Furthermore, it has recently been concerned that pertussis toxin toxoid obtained through the detoxification with the conventional agents such as formaldehyde may exhibit toxicity reversion during storage, thereby giving the vaccines containing the toxoid undesirable side effects (Pertussis Vaccine Workshop '87). It is therefore being recommended that the toxoid used be one having no substantial toxicity reversion, which may be determined, for example, by maintaining the toxoid at 37°C for a period of several weeks.

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The present invention seeks to overcome the above-mentioned problems and to provide a novel method for preparing pertussis toxin toxoid from pertussis toxin by which the resultant toxoid is completely devoid of toxicity and does not exhibit toxicity reversion while retaining the sufficient antigenicity.

DESCRIPTION OF THE INVENTION

Through extensive studies, the present inventors have found that, in the treatment of pertussis toxin with formaldehyde (formalin) for the preparation of the toxoid, the presence of specific amino acids provide sufficient inactivation of the toxin without possibility of the toxicity reversion.

Thus, according to the present invention there is provided a method for preparing pertussis toxin toxoid by a treatment of pertussis toxin with formaldehyde, which comprises carrying out said treatment in the presence of lysine or glycine in combination with one or more of amino acids selected from the group consisting of N-acetyl-DL-tryptophan, N-acetyl-D-tryptophan and N-acetyl-L-tryptophan.

In another aspect of the invention there is provided a pertussin toxoid exhibition, no toxicity reversion when maintained at 37°C for four weeks, and in which physiological and biological activities of pertussis toxin are inactivated while immunogenicity is retained.

In still another aspect of the invention there is provided a vaccine comprising an immunologically effective amount of a pertussis toxin toxoid of the invention, in association with a pharmaceutically acceptable carrier.

The present inventors previously suggested that the presence of amino acid(s) may be advantageous in detoxification of antigens which are regarded as having preventive effect against the infection with pertussis (Japanese Patent Application No. 58548/1983). However, in the earlier application no mention was made specifically regarding the combination of lysine with N-acetyltryptophan (N-acetyl-DL-tryptophan, N-acetyl-D-tryptophan or N-acetyl-L-tryptophan), or the combination of glycine with N-acetyltryptophan.

The present invention is based on the fact that the treatment of pertussis toxin with formaldehyde in the presence of N-acetyltryptophan together with lysine or glycine can produce toxoids which are much superior to those obtained by conventional detoxification methods including the ones described in the above-mentioned earlier application by the present inventors (Japanese Patent Appln No. 58548/1983) in which there are employed other amino acid(s) than those defined in the present invention. It is particularly notable that the pertussis toxin toxoid prepared by the method of the present invention does not exhibit substantial toxicity reversion even under severe conditions, as evidenced by an assay system recently developed for sensitive analysis.

The method of the present invention for the preparation of the toxoid is generally carried out with 0.2 to 1.2% of formalin for 5 to 50 μ g of pertussis toxin in terms of PN(protein

nitrogen)/ml, in which the concentration of N-acetyltryptophan is in the range of 0.1 to 10mM, the concentration of lysine is in the range of 5 to 200mM and the concentration of glycine is in the range of 5 to 200mM, at a temperature of 20 to 45°C for a treatment period of 3 to 30 days. Between lysine and glycine, lysine is preferable. The method of the present invention is practiced preferably with the formalin concentration being 0.3 to 1.0% (most preferably 0.6 to 0.8%), N-acetyltryptophan concentration being 0.2 to 4mM (most preferably 0.5 to 2mM), lysine concentration being 20 to 100mM (most preferably, 10 to 50mM), glycine concentration being 5 to 120mM (most preferably 20 to 40mM), and the temperature being 25 to 45°C (most preferably 37 to 42°C) for a period of 7 to 21 days (most preferably 7 to 10 days).

In the toxoid prepared by the method of the present invention, the various physiological and biological activities possessed by the pertussis toxin are reliably inactivated for detoxification, while the immunogenicity is retained as can be confirmed by the mouse potency test. What is more, the pertussis toxin toxoid obtained by the present invention does not demonstrate substantial change in the degrees of the physiological and biological activities and does not produce toxicity reversion, even when maintained at 37 °C for a period of several weeks. Accordingly, the method of the present invention for the preparation of the toxoid is of outstanding utility for providing efficacious and safe pertussis vaccines.

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The present invention will be more specifically described with reference to the following working example, which does not limit the scope of the present invention.

EXAMPLE

Pertussis toxin, 20 µg PN/ml (PN:protein nitrogen), was added with amino acid(s) as given in the table below. The mixture was added with formalin in an amount of 0.6 to 0.8% and the resultant was then kept at 40 °C for 7 to 21 days. After dialysis against PBS (M/75) to remove the formalin, pertussis toxin toxoids were obtained. Toxoid samples, 2 µg PN/ml, were each added with aluminum gel, 0.2mg/ml, to prepare vaccine solutions. Each vaccine solution was kept 4 °C for 4 weeks, or at 37 °C for 4 weeks. The vaccine solutions were then determined for the various physiological and biological activities, with the results as shown in the table.

Determinations of the physiological and biological activities were conducted in known ways described in the literature references given below:

Mouse Potency[MP]: Minimum Requirements of Biological Products in Japan 1981, Pharmaceutical Affairs Bureau, Ministry of Health of Japan, published by Association of Biological Manufacturers of Japan.

LPF activity (mouse LPF activity)[LPF]: ditto

HSF activity (mouse HSF activity)[HSF]: ditto

CHO-cell clustering activity (Chinese hamster ovary cell clustering activity)[CHO]: J. Biol. Stand., 13, 61-66(1985).

Skin vascular permeability [SVP]: Microbiol. Immunol., 31,
531-539(1987).

Blood glucose value [BGV]: Infect. Immun., 41, 137-144 (1983).

ADP-ribosylase activity (ADP-ribosyltransferase activity)
[ADP]: Infect. Immun., 46, 422-428 (1984).

The toxoids (Sample Nos. 1,2,8 and 9) prepared according to the method of the present invention exhibited no toxic activities while possessing high potencies, as can be seen from the data on the physiological and biological activities given in the table. Particularly notable is that the toxoids obtained by the present invention demonstrated no toxic activities and no toxicity reversion even when maintained at 37°C for four weeks. Such effect is particularly notable when N-acetyltryptophan and lysine are added.

In contrast, samples subjected to a single addition of N-acetyltryptophan, lysine or glycine (Samples Nos. 3,4 or 5) showed a tendency toward toxicity reversion as such toxoid samples still exhibited some extent of physiological and biological activities when maintained at 37°C. It will also be noted that the treatment with formalin alone (Sample No.6), as commonly practiced, does not provide satisfactory inactivation of the physiological and biological activities. This is particularly the case when the sample was maintained 37°C, since the results show that the substantial levels of the physiological and biological activities for toxicity are still remained.

Condition										Physiological and Biological Activities of Toxoids										MP
Sample No.	Amino Acid(s)	Amino Acid Con (mM)	Temp. (°C)	Formalin Con. (%)	Period (days)	L PF		HSF		CHO		SVD		BGV		ADP		(IPU/ml)		
						4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C			
1	N-acetyl-DL-tryptophan lysine	1.722 50	40	0.8	7	0.35	0.35	0.06	0.05	<0.25	<0.25	—	—	220	215	—	—	13.1		
2	glycine	1.722 44	40	0.8	7	0.35	0.35	0.06	0.05	<0.25	<0.25	—	—	215	190	—	—	14.0		
3	(control)	1.722	40	0.8	7	0.37	0.50	0.10	1.20	<0.25	<0.25	—	—	ND	ND	—	—	11.0		
4	(control)	50	40	0.8	7	0.42	0.50	0.06	0.60	<0.25	<0.25	—	—	ND	ND	—	—	8.0		
5	(control)	44	40	0.8	7	0.38	0.50	0.06	0.75	<0.25	<0.25	—	—	ND	ND	—	—	11.5		
6	No amino acid	0	40	0.8	7	0.35	0.85	0.09	1.50	2.3	270	+	+	189	185	+	+	12.2		
(7)	Saline (for Biol. Activity Control)	0	0	0	0	0.37	0.37	0.06	0.06	<0.25	<0.25	—	—	217	210	—	—	0		
8		0.574 10	40	0.8	14	0.35	0.35	0.06	0.06	<0.25	<0.25	—	—	220	217	—	—	12.5		
9		0.574 20	25	0.6	27	0.35	0.35	0.06	0.05	<0.25	<0.25	—	—	210	215	—	—	12.7		

ND : No experiment was done.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method for preparing pertussis toxin toxoid by a treatment of pertussis toxin with formaldehyde, which comprises carrying out said treatment in the presence of lysine or glycine in combination with one or more of amino acids selected from the group consisting of N-acetyl-DL-tryptophan, N-acetyl-D-tryptophan and N-acetyl-L-tryptophan.

2. A method as claimed in claim 1, in which said formaldehyde comprises formalin in an amount of 0.2 to 1.2% for 5 to 50g of pertussis toxin in terms of PN (protein nitrogen)/ml, the concentration of N-acetyltryptophan is in the range of 0.1 to 10mM, and the treatment is carried out in the presence of lysine in a range of 5 to 200mM, at a temperature of 20 to 45°C for a treatment period of 3 to 30 days.

3. A method as claimed in claim 1, in which said formaldehyde comprises formalin in an amount of 0.2 to 1.2% for 5 to 50g of pertussis toxin in terms of PN (protein nitrogen)/ml, the concentration of N-acetyltryptophan is in the range of 0.1 to 10mM, and the treatment is carried out in the presence of glycine in a range of 5 to 200mM, at a temperature of 20 to 45°C for a treatment period of 3 to 30 days.

4. A method as claimed in claim 2, in which the formalin concentration is 0.3 to 1.0%, N-acetyltryptophan concentration is 0.5 to 2mM, lysine concentration is 10 to 50mM, and the temperature is 37 to 42°C for a period of 7 to 10 days.

5. A method as claimed in claim 3, in which the formalin concentration is 0.3 to 1.0%, N-acetyltryptophan concentration is 0.5 to 2mM, glycine concentration is 20

to 40mM, and the temperature is 37 to 42°C for a period of 7 to 10 days.

6. A method as claimed in claim 1, in which said treatment is carried out in the presence of lysine in a range of 5 to 200 mM.

7. A pertussis toxin toxoid exhibiting no toxicity reversion when maintained at 37°C for four weeks, and in which physiological and biological activities of pertussis toxin are inactivated while immunogenicity is retained, produced by a method of claim 1, 2, 3, 4, 5 or 6.

8. A vaccine comprising an immunologically effective amount of a pertussis toxin toxoid of claim 7, in association with a pharmaceutically acceptable carrier.