CONVENTION APPLICATION FOR A PATENT

BEHRINGWERKE AKTIENGESELLSCHAFT,
of Marburg/Lahn,
Federal Republic of Germany.

hereby apply for the grant of a Patent for an invention entitled:

"ATOXI, IMMUNOGENIC PRODUCT OF TETANUS TOXIN"

which is described in the accompanying complete specification. This application is a Convention application and is based on the application numbered (a)

P 26 34 584.6 for a patent or similar protection made in the Federal Republic of Germany on 31st July, 1976.

My address for service is Messrs. Edwd. Waters & Sons, Patent Attorneys, 50 Queen Street, Melbourne, Victoria, Australia.

DATED this 26th day of JULY 1977.

By

[Signature]

L. C. GEBHARDT
Reg'd. Patent Attorney
COMMONWEALTH OF AUSTRALIA

Patents Act 1952

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION UNDER PART XVI. FOR A PATENT.

In support of the Convention application made under Part XVI. of the Patents Act 1952 by BEHRINGWERKE AKTIENGESELLSCHAFT of Marburg/Lahn, Federal Republic of Germany for a patent for an invention entitled: "ATOXI, IMMUNOGENIC PRODUCT OF TETANUS TOXIN"

Kurt Dräger
We,
and Max Schönenberger

13, Auf der Hube, Marburg (Lahn),
Federal Republic of Germany

30, Am Berg, Marburg (Lahn),
Federal Republic of Germany

do solemnly and sincerely declare as follows:

1. We are authorized by BEHRINGWERKE AKTIENGESELLSCHAFT the applicant for the patent to make this declaration on its behalf.

2. The basic application as defined by Section 141 of the Act was made at München in the Federal Republic of Germany under No. P 26 34 584.6 on the 31st day of July 1976 by BEHRINGWERKE AKTIENGESELLSCHAFT.

3. Torsten Bertil Helting, 24, Oberer Eichweg, Marburg/Lahn Federal Republic of Germany is/are the actual inventor(s) of the invention and the facts upon which BEHRINGWERKE AKTIENGESELLSCHAFT is entitled to make the application are as follows:

The said BEHRINGWERKE AKTIENGESELLSCHAFT is the assignee of the said Torsten Bertil Helting.

4. The basic application referred to in paragraph 2 of this Declaration was the first application made in a Convention country in respect of the invention the subject of the application.

DECLARED at Marburg/Lahn, Federal Republic of Germany this 12th day of July 1977

To the Commissioner of Patents

BEHRINGWERKE
Aktiengesellschaft

(Dräger) (Schönenberger)
Processes for producing the toxin derivative include treatment with aldehydes and proteinases.

CLAIM

1. An atoxic, immunogenic product from tetanus toxin, which is characterized by the following parameters:
   a. Sedimentation constant $S_0^{20,W} 5.66 \pm 0.34$
   b. Immunologic reaction with tetanus antitoxin: partially identical with that of tetanus toxin.
   c. Immunologic reaction with fragment C: not identical.
   d. Molecular weight, determined by a gel electrophoresis in sodium dodecylsulfate, of 95 000 \( \pm \) 5 000.
   e. Cleavable by reduction into a sub-unit having a molecular weight of 45 000 \( \pm \) 2 500 and a sub-unit immunologically identical with the known derivative of the light chains of the tetanus toxin, having a molecular weight of 48 000 \( \pm \) 2 500 (each time determined in the sodium dodecylsulfate electrophoresis).
Name of Applicant: BEHRINGWERKE AKTIENGESELLSCHAFT

Address of Applicant: Marburg/Lahn, Federal Republic of Germany.

Actual Inventor: Torsten Bertil Helting

Address for Service: EDWD. WATERS & SONS, 50 QUEEN STREET, MELBOURNE, AUSTRALIA, 3000.

Complete Specification for the invention entitled: "ATOXI, IMMUNOGENIC PRODUCT OF TETANUS TOXIN"

The following statement is a full description of this invention, including the best method of performing it known to: US
The present invention relates to an atoxic, immunogenic product of tetanus toxin.

The invention provides an atoxic, immunogenic product which may be obtained from tetanus toxin by means of a proteinase. It also provides a tetanus vaccine containing the novel product.

As has already been known, tetanus toxin is highly toxic towards mammals and also humans and must be detoxicated, in order to be used as an immunizing agent. It has been known to detoxicate the tetanus toxin by treating it with formaldehyde.

It has also been known that an atoxic, immunogenic product can be obtained by a treatment of tetanus toxin with a proteinase. This atoxic, immunogenic product is labelled as fragment C of the tetanus toxin in scientific literature.

It was a surprising fact which could not have been foreseen that in the treatment of tetanus toxin with a proteinase, preferably with papain, a further substance having especially advantageous properties is formed which can be characterized chemically in a definite manner and which may be isolated according to known biochemical processes.

The particularly advantageous properties of the product termed fragment B include the absence of toxicity and the immunogenicity of the same. These properties make the product suitable as a component of vaccines against tetanus.

The present invention therefore provides an atoxic, immunogenic product which may be obtained from tetanus toxin by treating the same with a proteinase, preferably with papain, which product is characterized by the following parameters:

1. Sedimentation constant $S_{20W}^0$ 5.66±0.34
2. Immunologic reaction with tetanus antitoxin:
partially identical with that of tetanus toxin.

3. Immunologic reaction with fragment C:
   not identical.

4. Molecular weight, determined by a gel electrophoresis in sodium dodecylsulfate, of 95 000 ± 5 000.

5. Cleavable by reduction into a sub-unit having a molecular weight of 45 000 ± 2 500 and a sub-unit immunologically identical with the known derivative of the light chains of the tetanus toxin, having a molecular weight of 48 000 ± 2 500 (each time determined in the sodium dodecyl-sulfate electrophoresis).

The variations shown in the parameters are due to the limits of error within the methods of determination.

The sedimentation constant was determined in an aqueous solution of 0.2 molar NaCl, 0.02 molar Na₂HPO₄, 0.03 molar NaH₂PO₄ at a pH of 6.8 according to T. Svedberg and K.O. Pedersen, "The Ultra-centrifuge", The Clarendon Press, Oxford, 1940, in an overlayer cell of an analytical ultracentrifuge, with reference to Vinograd, Proc. Acad. Sci. USA, 49, 902 (1963). The overlayering action of the test solution containing the product of the invention was effected on a 1 molar NaCl solution having a pH value of 7.0.

The immunologic reactivity was examined while using antisera, which were obtained by the immunisation of rabbits with tetanus toxoid or the fragment B of the invention. Use was made of the known double diffusion technique. By means of this technique it can be shown that the formerly described fragment C is clearly distinguished from the fragment B of the invention.

As could further be shown, fragment B can be dissociated into

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two fragments, one of which shows the same immunologic behavior as the derivative of the light chain described in Patent Application No. P 25 57 047.

The gel electrophoresis in a 7% polyacrylamide gel with the use of sodium dodecylsulfate as a component of the electrophoresis buffer has been carried out according to K. Weber and M. Osborn, J. Biol. Chem., Vol. 244, 4406–4412 (1969). Prior to carrying out the gel electrophoresis, the samples to be analyzed were mixed in an aqueous solution with sodium dodecylsulfate up to a concentration of 1% and were kept for 1 minute in boiling water. The calculation of the molecular weight is effected by a comparison with standard substances for determining the molecular weight (Aldolase and Katalase which may be obtained by Messrs. Boehringer, Mannheim, No. of Cat. 15 575 - "Eichproteine Größe II"). The calculation method has been indicated in the above-cited paper by Weber and Osborn.

The novel atoxic, immunogenic product labelled fragment B of the tetanus toxin may be obtained according to the process described in Patent Application No. P 25 10 987, by treating tetanus toxin with a proteinase. According to a preferred embodiment, fragment B is obtained by the treatment of tetanus toxin, which may be produced from culture filtrates of Clostridium Tetani by way of ion exchange chromatography, with papain, preferably in the form of a substance being bound to a carrier, in the presence of a reducing agent.

In this process it has proved to be advantageous to choose an incubation temperature which is higher than usual, i.e. between 30 and 60° C, preferably between 45 and 55° C. By this measure it is possible to increase the yield of the product of
the invention. Following the proteolytic action on the tetanus
injection, the enzyme bound to a carrier is eliminated from the re-
aaction mixture, suitably by way of centrifuging, and the remain-
ing solution is subjected to a fractionation through a molecular
sieve. In this process the use of dextran cross-linked with
epichlorohydrin has proved to be advantageous, for example
Sephadex(R) G 100 of Firm Pharmacia, Uppsala or Ultrogel(R) of
LKB, Bromma or bio-gel p(R) of Bio-Rad Laboratories, Richmond,
Calif.

In case there is used a soluble enzyme preparation, the
separation of the enzyme is effected in the process of frac-
tionation through the molecular sieve.

Once fragment B has been obtained in this manner, an anti-
serum may be prepared with the same by the immunization of test
animals with fragments B, and by means of said antiserum it is
then possible to obtain an immuno-adsorbing agent in common
manner. This agent serves in its turn to extract fragment B in
a particularly pure form from the incubation mixture of said
fermentation solution.

It is also possible to eliminate fragment C or tetanus toxin
from a mixture with fragment B by immuno-adsorptive measures.
For this purpose, an antiserum against the known fragment C is
at first prepared. By means of this antiserum, an immuno-ad-
sorbing agent can be obtained in known manner. Said agent is
suitable in its turn to eliminate the remainder of fragment C
and of tetanus toxin from fragment B, since fragment C as well
as tetanus toxin react with the anti-fragment C-serum, however,
fragment B does not.

Other processes of isolation, as they are known to the man
skilled in the art for separating proteins having a different electric charge, are also successful. They include above all electrophoretic processes as well as processes of ion exchange chromatography.

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The pure fragment B from tetanus toxin which may be obtained in this manner proves to be non toxic as compared against the tetanus toxin. Whereas \(30 \times 10^6\) min. lethal doses are detected per mg of the tetanus toxin, fragment B has 5 min. lethal doses per mg. With regard to the toxic symptoms, fragment B is seen to be completely different from tetanus toxin; it does not show the symptoms characterized by spastic paralysis. In spite of the reduction of the toxicity to \(1:6 \times 10^6\) it is advantageous to treat fragment B with an aldehyde, as has been described in the following.

For this process the product obtained by means of proteinases is treated after its isolation with a protein concentration of about 1 mg of protein per ml or less in a buffer solution having a pH value of from 6.0 to 8.5, preferably 7.8, and a molarity in the range of from 0.01 to 0.2 mole with 0.015 to 0.3 mole of an aliphatic mono- or dialdehyde of 1 to 6 carbon atoms, preferably formaldehyde, during 14 to 28 days at a temperature in the range of from 20 to 37°C. If desired, the product may be subjected to a dialysis of 10 to 20 hours, preferably 15 hours, against a physiologically acceptable solution, such as 0.15 molar sodium chloride, and/or it may be filtered under sterile conditions. For preparing the vaccine, the product is suitably also mixed with an adjuvant, for example, aluminum hydroxide. By dilution with a physiologically acceptable solution, such as 0.15 molar sodium chloride solution, the con-
centration is adjusted to the desired antigen content.

These vaccines may be used by themselves, but also in combination with other vaccines. For combination there are suitable, above all, diphtheria toxoid, pertussis immunogenic agents, poliomyelitis viruses, or measles viruses. By way of adsorption tests it can be shown that about one half of all protecting antibodies directed against tetanus toxin are directed against fragment B.

The following Examples serve to further illustrate the present invention.

EXAMPLE 1:

Clostrium-tetani are fermented in a latham medium. The toxin formed in this process is adsorbed at diethylaminoethyl cellulose ion exchangers and is eluted by a gradiently rising molarity of a phosphate buffer having a pH value of 7 from 0.01 mole to 0.4 mole. The fractions containing the toxin are again separated by chromatography in a column which is filled with Sephadex(R) G 100, and the fractions containing the toxin are extracted and combined.

2.5 Grams of tetanus toxin in a solution having a final concentration of 15 mg of tetanus toxin/ml of a 0.1 molar phosphate buffer of a pH of 6.5 containing 0.01 mole of Na₂EDTA and 0.001 mole of cystein-HCl are mixed with 40 mg of papain. The papain contains 30 units of enzymatic activity/mg of substance. At first, the mixture is maintained for 1 hour at 45°C, and thereafter for another 2 hours at 55°C.

For the separation of the tetanus toxin there may be used preferably also papain bound to a carrier.

For this purpose, a solution of 500 ml of papain dialyzed
against a 0.1 molar Na$_2$CO$_3$ buffer of a pH of 10.0 is combined with 50 mg/ml of an agarose gel activated with cyanobromide and the mixture is maintained for 24 hours at 4° C. The reaction product washed several times with a solution containing 4 moles of urea and 0.5 mole of NaCl per liter is used in a manner analogous to that of the soluble enzyme for the proteolytic separation of the tetanus toxin.

Upon cooling of the mixture, the volume is concentrated by means of an ultrafilter, and the mixture is then introduced into a column of a length of 10 x 100 cm filled with Sephadex$^{(R)}$ G 100. The elution is effected with a 0.1 molar tris(hydroxy)methylamino methane-hydrochloric acid buffer having a pH value of 8.0 and containing 1 mole of NaCl.

During the elution, the adsorption in the range of the wave length of 280 nm is measured continuously. The fractions showing an adsorption in this range are collected separately. There are formed 4 fractions, the first of which representing a double peak.

In the course of a repeated chromatography carried out under the same conditions, the two peaks are separated, and finally the second peak is isolated. This latter peak contains the atoxic, immunogenic product of the invention, the fragment B from tetanus toxin.

The fragment B of the invention can be obtained in a particularly simple manner, if an antiserum directed against the known fragment C (10 ml with 1 000 IU/ml) is bound in common manner to agarose activated by BrCN, and a preparation of fragment B which has been partially purified by gel chromatography is subsequently mixed with such an immuno-adsorbing
agent. After 60 minutes of stirring, the gel is separated together with the impurities bound to it, and fragment B is obtained by another gel chromatography.

It is also possible to neutralize the impurities by adding anti-fragment C-antiserum (IgG fraction) and to separate the immunizing complex compounds from fragment B by way of gel chromatography.

Finally, fragment B of the invention may also be obtained if an immuno-adsorbing agent for fragment B is prepared via an antiserum, with the aid of a fragment B once prepared. For this purpose, known processes may be applied. By means of the immuno-adsorbing agent, fragment B is adsorbed selectively from the mixture with other reaction products from the proteinase treatment of tetanus toxin, whereupon it may be eluted selectively from the immuno-adsorbing agent.

**EXAMPLE 2:**

Fragment B is diluted with 0.1 molar phosphate buffer solution of a pH of 6.5 to 200 μg of protein per ml, mixed with 0.06 % formaldehyde and allowed to stand at 37°C for 21 days. Thereafter, the solution is dialyzed for 16 hours against several times its volume of 0.15 molar sodium chloride solution and is then processed in common manner into a vaccine.
THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An atoxic, immunogenic product from tetanus toxin, which is characterized by the following parameters:
   a. Sedimentation constant $S_{20w}^0 = 5.66 \pm 0.34$
   b. Immunologic reaction with tetanus antitoxin:
      partially identical with that of tetanus toxin.
   c. Immunologic reaction with fragment C:
      not identical.
   d. Molecular weight, determined by a gel electrophoresis in sodium dodecylsulfate, of 95 000 \pm 5 000.
   e. Cleavable by reduction into a sub-unit having a molecular weight of 45 000 \pm 2 500 and a sub-unit immunologically identical with the known derivative of the light chains of the tetanus toxin, having a molecular weight of 48 000 \pm 2 500 (each time determined in the sodium dodecylsulfate electrophoresis).

2. Process for the preparation of the atoxic, immunogenic product as claimed in claim 1, which comprises extracting the product which may be obtained by treating tetanus toxin with a proteinase, by a fractionation through a molecular sieve, an immuno-adsorption process, an electrophoresis and/or by ion exchange chromatography.

3. Process as claimed in claim 2, which comprises treating the product at a pH value of from 0.6 to 8.5 with from 0.015 to 0.3 mole of an aliphatic mono- or dialdehyde with 1 to 6 carbon atoms for 14 to 28 days at a temperature in
the range of from 20 to 37° C.

4. A product, which is characterized by the parameters of a product to be obtained according to claim 3.

5. Use of the product as claimed in claim 1 as an essential component in a vaccine against tetanus.

6. Use of the product as claimed in claim 4 as an essential component in a vaccine against tetanus.

7. A tetanus vaccine which contains as active ingredient a product as claimed in claim 1.

8. A tetanus vaccine which contains as active ingredient a product as claimed in claim 4.

In this [26th] day of July 1977.

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