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Paper A(Ch)

Chemistry

This paper comprises:

LETTER FROM THE APPLICANT

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Dear Mr. Patent attorney,

[001] We are a small Biotech company producing protein compositions. In this context we have made a substantial invention which is extremely valuable to us. Please file a patent application for the matter we invented as soon as possible. Note that it is company policy not to pay any claims fees.

[002] Attached is a description of our invention and also two prior art documents which appear to be of relevance to our invention. Document D1 discloses a method comprising the same steps as our method. This is unfortunate as we would like to also gain protection for this method. Knowing, however, that novelty is one of the requirements to obtain a patent before the European Patent Office, it seems very unlikely that this method can be protected. Should you see, however, a possibility of nevertheless gaining protection for this method, then please ensure that this is done. Document D2 discloses compositions comprising the same proteins as those described in the present invention.

[003] We developed a process useful for finding solutions in which proteins keep their natural structure (stabilising solutions). If possible, we also wish to gain protection for stabilising solutions found by the said process.

[004] Cholera is a contagious disease widely distributed in the world. The principal symptom of cholera is diarrhea causing severe dehydration and loss of electrolytes. If untreated, 50 - 60 % of infected patients die.

[005] Cholera is caused by bacteria which release proteins into the human intestinal tract. These proteins belong to the family of Bacterial Diarrhea Proteins (BDP). This family of proteins comprises three types of proteins, each respectively containing one Alpha (A) and two, four or five Beta (B) subunits. The said three types of BDP proteins are therefore named AB2, AB4 and AB5. Typical AB5-type proteins are EcT (E. coli - Toxin) and CvT (Cholera- vibrio - Toxin).

[006] While it is generally accepted that AB5-type proteins are causative for cholera, their mechanism of action remains to be elucidated. Research in this field has been difficult because AB5-type proteins are highly instable and rapidly become non-functional. “Non-functional” means in this context that the protein complex consisting of one A and five B subunits has disintegrated and thus lost its natural three-dimensional structure. In this disintegrated state, the AB5-type protein fails to produce its natural biological effects and cannot be used for biochemical analysis. For this reason, AB5-type proteins are usually stored in dried form. For use, the protein is dissolved in a standard saline solution, kept at 4°C and rapidly used.

[007] There is thus a need to replace the standard saline solution currently used by a stabilising solution in which the AB5-type protein retains its natural protein complex structure for an extended period of time.

[008] We have developed a method to determine the proportion of complexed AB5 protein in a sample. This is achieved by submitting a sample of an AB5-type BDP protein to column chromatography using, as support material, hydroxylated polymethacrylate (HyPM) having free carboxyl groups.

[009] In our experiments we used the Ultrahydrogel-250 column (Waters Ltd) in the format of a High-Performance Liquid Chromatography (HPLC) column. The Ultrahydrogel-250 has a particle size of 6 μm and a porosity of 250 nm.

[010] For separation of complexed and disintegrated AB5-type proteins on the above support material, the following buffer must be used to elute the proteins: Tris-HCl 200 mM + Na_2SO_4 100 mM at pH 7.2. A still acceptable resolution is possible if the pH of this buffer varies from 6.8 to 7.6. Beyond these boundaries no meaningful results could be obtained.

[011] Proteins were detected during chromatography by UV absorbance spectroscopy as is standard in the art. Only two peaks were detectable, one corresponding to the B5 unit and one corresponding to complexed AB5. We were thus able to detect and quantify the AB5-type protein in its complexed state and also when disintegrated.

[012] The stability of the AB5-type protein may be expressed in form of a Stability Factor (SF). The SF was calculated as the ratio of the amount of complexed AB5-type protein divided by the total amount of AB5 + B5 (in %). The AB5-type protein was deemed stabilized if the SF was more than 70 %, preferably more than 80 %, even more preferably more than 90 %.

[013] The above separation method permits, for the first time ever, quantitative measurement of protein complex stability of an AB5-type protein. Having this method hand, we established a process to find stabilising solutions that resulted in an enhanced stability of an AB5-type protein in comparison with the standard saline solution currently used.

[014] The term “stabilising solution” refers in this context to an aqueous solution which contains one or more stabilising agents that help to maintain the AB5-type protein in its complexed and thus biologically active form.

[015] Screening for stabilising agents is routine for the skilled person in the field of protein formulation. By way of example, the candidate stabilising agent may be selected from sugars, detergents or amino acids. The method must comprise the steps of incubating the AB5-type protein in a test stabilising solution and of measuring the stability of the AB5-type protein complex as described above.

[016] The incubation of the sample may last from hours to several days, weeks or even months. The incubation may take place under cooled conditions (such as 4 °C) or at ambient temperature (between 20 °C and 25 °C). During incubation, the sample may either be kept in static condition or may be shaken. The latter may be used to accelerate complex disintegration and thus shorten incubation time.

[017] An AB5-type protein in a stabilising solution may further be tested for biological activity. This can be done by numerous in vivo or in vitro tests well known to the skilled person and described in, for example, Yamaha (1992) J. Prot. Technol..

Examples

Example 1: Separation of AB5 and B5 of CvT by chromatography

[018] In this experiment, commercially available dried CvT was dissolved in the standard saline solution and incubated under the conditions indicated in Table 1. Then, the sample was loaded onto the HPLC-Ultrahydrogel-250 column described above and protein was eluted using the following buffer: Tris-HCl 200 mM + Na_2SO_4 100 mM pH 7.0. Protein peaks were detected, quantified and the Stability Factor (SF) calculated.

Table 1

	Stability Factor (%) at incubation temperature	
Incubation conditions	**4 °C**	**22 °C**
1 hour, static	75	56
12 hours, static	61	33
24 hours, static	45	23
48 hours, static	37	18

[019] The data in Table 1 show that the protein rapidly disintegrates when dissolved in the standard saline solution. Storage at ambient temperature substantially aggravates this problem.

Example 2: Screening for AB5-type protein stabilising solutions

[020] Solutions were tested for their stabilising effect on an AB5-type protein complex. We used CvT as AB5-type protein. CvT was used at 0.8 – 2.0 mg/ml. CvT was incubated at 4 °C for 12 hours in different candidate stabilising solutions under the conditions as indicated in Table 2. Following incubation, the samples were analysed by the method of example 1. The candidate stabilising solutions tested were taken from a commercially available Protein-Stabilisation-Test Kit.

Table 2

Candidate stabilising solution		Stability Factor	
		static	**shaken**
1	PBS	55	45
2	PBS, galactose 0.1 mM	61	55
3	PBS, 0.25 wt.% CHAPS	95	89
4	Phosphate buffer, pH 7.4	65	54
5	Phosphate buffer, pH 7.4, galactose 0.2 M	64	52
6	Phosphate buffer, pH 7.4, L-arginine 0.4 M	88	82
7	Acetate buffer, pH 5.5, NaCl 300 mM	62	43
8	Citric Acid buffer, pH 6.5	65	38
9	Tris buffer, pH 7.5, 1 mM EDTA	59	41
10	Tris buffer, pH 7.5, L-arginine 0.4 M	59	39
11	Control: standard saline solution	61	42

[021] The data in Table 2 demonstrate that two solutions (no. 3 and 6) are particularly effective in stabilising CvT. These stabilising solutions were chosen for further analysis.

Example 3: Effect of L-Arginine and CHAPS on CvT stability

[022] The stabilising effect of stabilising solutions no. 3 and 6 was examined over time (1, 2, 10, 30, 60 and 90 days) under static conditions. CvT was kept at a protein concentration of 2.0 mg/ml and was maintained at 2 - 8 °C in the stabilising solution. Protein complex stability was detected by the method of example 1 and the Stability Factor was calculated.

Table 3

Stabilising solutions	**Incubation time (days)**					
	1	**2**	**10**	**30**	**60**	**90**
Phosphate buffer, pH 7.4 + 50 mM L-Arginine	86.5	87.5	88.8	87.0	85.5	84.0
Phosphate buffer, pH 7.4 + 100 mM L-Arginine	88.8	87.2	85.6	90.5	83.4	82.2
Phosphate buffer, pH 7.4 + 200 mM L-Arginine	86.3	88.3	83.7	83.5	81.2	82.4
Phosphate buffer, pH 7.4 + 400 mM L-Arginine	85.8	88.8	88.3	87.2	85.3	83.9
PBS + 0.05 wt.% CHAPS	85.5	87.5	86.8	87.0	86.5	85.0
PBS + 0.15 wt.% CHAPS	90.8	89.2	88.6	88.5	88.4	88.2
PBS + 0.25 wt.% CHAPS	93.3	91.3	90.7	90.5	90.2	89.4
PBS + 0.35 wt.% CHAPS	90.7	88.8	88.3	87.2	90.3	87.9

[023] The data in Table 3 demonstrate that both CHAPS and L-Arginine in their respective buffers stabilize AB5-type proteins as exemplified by CvT for up to 90 days. This stabilising effect is observed over a wide range of pH-values (data not shown). Increasing the amount of L-Arginine beyond 50 mM does not improve protein complex stability. Satisfactory stabilization is obtained when using L-Arginine in an amount of at least 10 mM. The protein complex is stabilized if at least 0.05 wt.% of CHAPS is present. Better results, however, were obtained with at least 0.15 wt.% CHAPS.

Best regards,

Peter St. John

Document D1

Isolation of homo-duplex protein VIP2 from S. Echinaceae

Background

[001] The VIP2 protein (Very Important Protein) consists of two identical VIP-subunits forming biologically active VIP2. This protein represents an important component of the cell signalling pathway of S. Echinaceae.

[002] Peters et al. (1998) provided an optimised protocol of VIP2 purification from cellular extracts of S. Echinaceae. This protein purification protocol was, however, not satisfactory as the level of protein purity obtainable (approx. 94 %) was insufficient.

[003] We set out to improve on what has been achieved so far. Starting from the protein extract obtained by implementing the method of Peters et al. (1998), we performed an extensive analysis of numerous possibilities to eliminate the remaining contaminants from the VIP2 containing sample. We now present a simple procedure extending the strategy established by Peters et al. (1998) to enrich VIP2 to purity levels of at least 99 %.

Materials and Methods

[004] As starting material we used a VIP2 containing sample prepared according to the method described by Peters et al. (1998).

[005] In preliminary experiments, numerous commercially available High-Performance Liquid Chromatography (HPLC) columns were tested. Eventually, a break-through was achieved using a commercially available hydroxylated polymethacrylate (HyPM) material having free carboxyl groups as support material (Ultrahydrogel-250, available from Waters Ltd.).

HPLC column chromatography

[006] Buffer: Tris-HCl 200 mM + Na_2SO_4 100 mM at pH 7.2; flow: 0.5 ml/min; detection: UV absorbance spectroscopy; column: Ultrahydrogel-250 (Waters Ltd.); support material: Hydroxylated polymethacrylate (HyPM) with free carboxyl groups; particle size: 6 μm; porosity: 250 nm.

[007] The chromatogram of the proteins eluted using this method showed a single peak corresponding to the VIP2 dimer. A range of low molecular weight contaminants that were clearly separated from VIP2 was also detected. VIP2 identity was confirmed by mass-spectrometry.

Conclusion

[008] We report for the first time a purification protocol for VIP2 that permits significant improvement in the level of VIP2 protein purity over the reference protocol published by Peters et al. in 1998. Having achieved a level of protein purity above 99 %, the time has come for in-detail study of the biology and biochemistry of this protein which is important in cellular signalling in S. Echinaceae.

Document D2

Detoxified mutants of cholera toxin (CvT)

[001] Cholera is a contagious disease widely distributed in the world. It is caused by microorganisms such as Cholera vibrio (C. vibrio). The principal symptom is severe diarrhea resulting in life threatening dehydration and loss of electrolytes. Although cholera can be effectively cured by controlled and intense rehydration further research of the disease remains necessary.

[002] C. vibrio causes diarrhea through the secretion of Cholera-vibrio Toxin (CvT). CvT belongs to the family of bacterial diarrhea proteins (BDP) which comprises three types of proteins, each containing one Alpha (A) and two, four or five Beta (B) subunits. The three types of proteins are therefore named AB2, AB4 and AB5.

[003] The use of AB5-type proteins in animal models such as mice, rats and rabbits has proven difficult due to the inherent toxicity of these proteins. To overcome this problem, AB5-type proteins have been chemically altered by cross-linking agents aiming to reduce their level of toxicity while still causing diarrhea.

[004] In the present invention we provide an alternative solution to the problem. We discovered that CvT can be detoxified, while maintaining its activity to cause diarrhea, if at least one of the following amino acids in the A-subunit of CvT is mutated: Val-53 or Ser-97.

[005] As used herein, the term “detoxified” means that the AB5-type protein exhibits substantially lower toxicity than the naturally occurring protein. Ideally the level of toxicity should be less than 0.01 % of that of the naturally occurring protein.

Example: Detoxified CvT

[006] The CvT gene was cloned and mutants generated according to standard procedures. The naturally occurring and mutant proteins were produced and toxicity was evaluated using the process described by Jensen et al. in ToxProt (1995).

[007] Proteins showing no or only minimal toxicity were then tested for their ability to cause diarrhea in mice. For this purpose, the proteins were purified, precipitated and dried as is conventional for AB5-type proteins. The dried protein was dissolved in an aqueous buffer consisting of PBS containing 0.25 wt.% CHAPS and the solution obtained was immediately administered at a dose of 2 mg/kg.

[008] Most of the detoxified proteins retained their ability to cause diarrhea in mice. The CvT mutants named CvT-5, CvT-23 and CvT-28 showed no toxicity in comparison to the naturally occurring CvT protein but were strongly active as diarrhea causing agents.

[009] These mutant CvT proteins carried the following changes in comparison to non-mutated, naturally occuring CvT:

CvT–mutant	Mutation
CvT-5	Val-53 to Asp-53
CvT-23	Ser-97 to Glu-97
CvT-28	Ser-97 to Lys-97

Claims:

1. Cholera-vibrio Toxin (CvT) wherein the amino acids at positions Val-53 and/or Ser-97 in the A subunit are replaced with another amino acid.

2. CvT according to claim 1 wherein Val-53 is replaced by Asp-53 and/or wherein Ser-97 is replaced by Glu-97 or Lys-97.

3. A composition comprising the protein of claims 1 or 2 in an aqueous solution.

4. The composition of claim 3 wherein the aqueous solution comprises PBS + 0.25 wt.% CHAPS.