Candidate's Answer

Stabilisation of AB5-type proteins

Technical field

The present invention relates to separation and detection of complexed AB5-type proteins. The present invention also relates to identifying solutions for stabilising complexed AB5-type proteins, and to stabilising solutions themselves.

Prior art

~~[004]~~ *1 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]~~ *2 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.*

D1 discloses a procedure for enhancing purity levels of the VIP2 protein. The process involves submitting an already purified sample to HPLC using hydroxylated polymethacrylate having free carboxyl groups. The buffer is Tris-HCl 200 mM + Na_2S0_4 100 mM at pH7.2. Using this method VIP2 of 99% purity was obtained.

D1 does not disclose purifying and separating AB5-type proteins. D1 does not mention these proteins. The VIP2 proteins of D1 are composed of two identical subunits.

D2 discloses detoxification of CvT (which is an AB5-type protein) through mutation of specific amino acids. D2 discloses that dried protein was dissolved in buffer containing 0.25 wt% CHAPS. The protein in solution was administered immediately.

D2 does not disclose anything about problems in stability of AB5-type proteins. Indeed, given that those proteins were administered immediately, stability would not have been a consideration.

Problem/solution

The problem solved by the present invention is the provision of solutions for enhancing stability of AB5-type proteins. The problem is solved by providing a method for identifying such solutions and by providing solutions themselves.

Neither D1 nor D2 teach or suggest that the methods and solutions would solve the problem. Neither D1 nor D2 mention stability of AB5-type proteins. Neither of those documents provide any incentive for developing methods/compositions (solutions) for storing the proteins.

Summary of the Invention

The invention therefore provides <claim 1>.

Preferably, the column has a particle size of 6µm and a porosity of 250nm. This allows separation of the proteins.

The invention also provides <claim 3>.

Preferably, the incubation takes place at 4°C or at 20-25°C . This reflects stability at ambient temperature.

Preferably the sample is shaken during incubation. This accelerates complex degradation and shortens the incubation time.

Preferably, the method further comprises testing for biological activity of the protein. This provides a further indicator of suitability of the solution for storage of the protein.

Preferably, the protein is CvT. The Examples show how stability of CvT is enhanced.

The invention further provides <insert claim 9>.

Preferably, <insert claim 10>. Effects are seen at this concentration range.

Preferably, the pH is 7.4. Again, high stability is seen at this pH.

The invention also provides <insert claim 12>.

Preferably <insert claims 13-15>.

Detailed description

[008] *We have developed a method to determine the proportion of complexed AB5-type 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 at 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 colled 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 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: Separtaion 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-HCI 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, glactose 0.2 M*	*64*	*52*
6	*Phosphate buffer, pH 7.4, L-arginine 0.4 M*	*88*	*82*
7	*Acetate buffer, pH 5.5, NaCI 300 mM*	*62*	*43*
8	*Citric Acid buffer, pH 6.5*	*65*	*38*
9	*Tris buffer, pH 6.5, 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. % CHAPS is present. Better results, however, were obtained with at least 0.15 wt.% CHAPS.*

CLAIMS

1. A method of detecting and quantifying the amount of complexed AB5-type protein compared to the disintegrated B5 unit in a sample, the method comprising:

   (a) submitting a sample of the proteins to column chromatography using hydroxylated polymethacrylate having free carboxyl groups as the support material;

   (b) eluting the complexed AB5-type protein and B5 units using a buffer comprising Tris-HCl 200 mM + Na2SO4 100 mM at pH 6.8-7.6; and

   (c) detecting and quantifying the amount of complexed AB5 and B5 unit by UV absorbance spectroscopy.

2. The method of claim 1, wherein the column has a particle size of 6 µm and a porosity of 250 nm.

3. A method of screening for stabilising solutions which enhance the stability of the AB5-type protein complex, the method comprising:

   (a) incubating the AB5-type protein in a test stabilising solution;

   (b) detecting and quantifying the amount of a complexed AB5-type protein compared to the disintegrated B5 unit according to the method of any one of claims 1-4;

   (c) calculating the stability factor, which is the ratio of the amount of complexed AB5-type protein divided by the total amount of AB5 + B5 (in %),

   wherein the stabilising solution is identified as enhancing the stability of the AB5-type complex if the stability factor is greater than 70%.

4. The method of claim 3, wherein the incubation takes place at 4 °C .

5. The method of claim 3, wherein the incubation takes place at 20-25 °C .

6. The method of claim 3, 4 or 5, wherein the sample is shaken during incubation.

7. The method of any one of claims 3-6, further comprising testing the AB5-type protein in the stabilising solution for biological activity.

8. The method of any one of the preceding claims, wherein the AB5-type protein is CvT.

9. Use of a solution comprising:

(a) phosphate buffer and at least 10 mM L-arginine; or

(b) PB5 + 0.05-0.20 wt.% CHAPS, or 0.30-0.35 wt.% CHAPS,

in stabilising an AB5-type protein.

10. The use according to claim 9, wherein the L-arginine is present at a concentration of 10-50 mM.

11. The use according to claim 9 or 10, wherein the pH of the phosphate buffer and L-arginine is 7.4.

12. A composition comprising an AB5-type protein and an aqueous solution of:

(a) phosphate buffer and at least 10 mM L-arginine; or

(b) PBS + 0.05-20, or 0.30-0.35 wt % CHAPS.

13. The composition according to claim 12, wherein the L-arginine is present at a concentration of 10-50 mM.

14. The composition according to claim 12 or 13, wherein the pH of the phosphate buffer and L-arginine is 7.4.

15. The use according to claim 9, 10 or 11, or the composition according to claim 12, 13 or 14, wherein the AB5-type protein is CvT.

Examination Committee I: Paper A Ch 2014 - Marking Details

Candidate No

Category		Maximum possible	Marks awarded	
			Marker	Marker
Independent claims	Method for determining the	15	15	15
Independent claims	Method for identifying a	20	18	18
Independent claims	Composition and/or uses	35	35	35
Dependent claims	Dependent claims	15	14	15
Description	Description	15	13	12
Total			**95**	**95**

Examination Committee I agrees on 95 marks and proposes the grade PASS