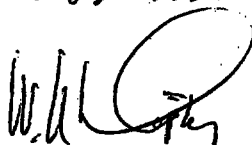


IN THE MATTER OF European  
Patent No. 0 219 874  
in the name of  
Boehringer Mannheim GmbH

I, Warwick Grenville Michael Shipley, of 40,  
Ridgmount Street, London, W.C.1., B.Sc., F.R.S.C.,  
C. Chem. F.I.L., F. Inst. Pet., am acquainted with  
the German and English languages, am a competent  
translator from German into English and certify  
that the attached is a true translation made by  
me into the English language of European Patent  
Specification No. 0 219 874.

Dated this 1st day of

July, 1993.



The invention concerns a process for the activation of gene-technologically produced, disulphide bridge-containing eukaryotic proteins after expression in prokaryotes.

5           In the case of the expression of heterologous proteins in prokaryotes, in the host cells these proteins often form inactive, sparingly soluble aggregates (so-called "refractile bodies") which, in addition, are also contaminated with proteins of the  
10 host cells. It is assumed that the formation of such "refractile bodies" is a result of the high protein concentration in the cell arising in the case of the expression. It is known that, in the case of the formation of large amounts of enzymes in the cell, the  
15 aggregation of the enzymes to insoluble, high molecular, mostly inactive particles takes place. Before such proteins can be used, e.g. for therapeutic purposes, they must consequently be purified and converted into their active form.

20           According to known processes, a reactivation of such proteins present as aggregates takes place in several steps (cf. e.g. R. Jaenicke, FEBS Federation of European Biochemical Societies, Vol. 52 (1979) 187 to 198; R. Rudolph et al., Biochemistry 18 (1979)  
25 5572 to 5575).

          In the first step, a solubilisation is achieved by addition of strong denaturing agents, for example guanidine hydrochloride or urea, in high concentration or by addition of strongly acidic agents, for example  
30 glycine/phosphoric acid mixtures. As further adjuvants, there have proved useful reducing SH reagents (e.g. dithioerythritol, DTE) and EDTA, for example in the renaturing of LDH. Insofar as the protein is contaminated by proteins of the host cells, as next step there  
35 follows a purification with per se known and usual

methods, e.g. gel or ion exchanger chromatography. Subsequently, it is highly diluted in order that the concentration of the denaturing agent becomes smaller. In the case of use of guanidine hydrochloride, it is  
5 thereby diluted to values below 0.5 mol/l. In the case of enzymes with free SH groups, the addition of agents protecting SH groups proves to be advantageous (cf. e.g. R. Jaenicke, Journal Polymer Science, Part C, 16 (1967) 2143 to 2160).

- 10 In EP-A-0114506, processes are described for the isolation, purification and reactivation of some heterologous expression products from bacterial cultures; for the reactivation, the solutions of the "refractile bodies" in a strong denaturing agent are
- 15 a) transferred directly into a solution of a weaker denaturing agent which is then subjected to oxidising conditions for the reformation of disulphide bridges;
- b) the protein is sulphonated, then transferred into a solution in a weak denaturing agent and the S-  
20 sulphonate groups are converted into -S-S- groups by treatment with a sulphhydryl reagent in its reduced and oxidised form, e.g. with GSH/GSSG; or c) the solution in a weak denaturing agent is treated directly with the sulphhydryl reagent, e.g. with GSH/GSSG. A  
25 typical example in which the above-discussed problems arises is t-PA.

In T.W. Odorzynski et al., 1979, The Journal of Biological Chemistry, 254(10), 4291-4295 and in C.F. Dudos et al., 1982, The Journal of Biological  
30 Chemistry, 257(16), 9866-9871, processes are described for the reactivation of natural proteins, such as e.g. trypsinogen.

The main component of the protein matrix of coagulated blood is polymeric fibrin. This protein  
35 matrix is dissolved by plasmin which is formed from

plasminogen via activation by the so-called plasminogen activators, e.g. by t-PA (tissue-type plasminogen activator). The enzymatic activity of natural t-PA or of t-PA obtained gene-technologically from eukaryotes (catalytic activation of plasminogen to plasmin) is very low in the absence of fibrin or fibrin cleavage products (FCP) but can be substantially increased in the presence of these stimulators (by more than the factor of 10). This so-called stimulatability of the activity is a decisive advantage of t-PA in comparison with other known plasminogen activators, such as urokinase or streptokinase (cf. e.g. M. Hoylaerts et al., J. Biol. Chem., 257 (1982) 291 to 2019; Nieuwenhuizen et al., Biochemica et Biophysica Acta 755 (1983) 531 to 533). Therefore, the factor of the stimulatability with BrCN cleavage products is variously given in the literature and given a value of up to 35.

A t-PA-like, non-glycosilated product is also formed in genetically manipulated prokaryotes (after introduction of the c-DNA); however, such a product does not have the stimulatability of the activity of a t-PA from eukaryotes. It is conceivable that the reason for this is that the redox conditions in the prokaryote cell differ in such a way from the eukaryote cells from which the gene originates that, ab initio, a non-active product is formed which, for example, could be due to the fact that numerous SS bridges which the natural active molecule contains are linked in a false way or are not even formed. However, for the therapeutic use of t-PA, there is necessary not only the enzymatic activity as such but, in addition, also its stimulatability. Regarding the fact that the prokaryote cells presumably do not provide the correct conditions in order to form the activity of eukaryotic proteins in the correct way, reference is made to other

substances in The EMBO Journal 4, No.3 (1985) 775 to 780.

According to EP-A-0093639, for the reactivation of t-PA, the cell pellets obtained from E. coli are  
5 suspended in 6 mol/l guanidine hydrochloride, treated with ultrasonics, incubated and subsequently dialysed for four hours against a solution of tris-HCl (pH = 8.0), sodium chloride, EDTA and Tween 80. After dialysis, it is centrifuged, whereby the plasminogen activator  
10 activity is to be found in the supernatant. t-PA renatured in this way is admittedly proteolytically active but shows no measurable stimulatability by BrCN cleavage products (BrCN-FCP) of fibrin according to the process described in J.H. Verheijen, Thromb.  
15 Haemostas., 48 (3), 260-269 (1982).

For the reactivation of denatured proteins, no generally usable process is known from the state of the art; this applies quite especially for t-PA because the native protein possesses a very complex structure;  
20 it contains a free thiol group and 17 SS bridges which theoretically can be linked in  $2.2 \times 10^{20}$  different ways, whereby only one structure corresponds to the native state. The processes known from the state of the art for the reactivation of t-PA admittedly lead  
25 to a proteolytically active t-PA but which shows no measurable stimulatability; an activation process which leads to stimutable t-PA is not known.

Therefore, it is the task of the present invention to make available a process for the complete  
30 activation of gene-technologically produced, heterologous, disulphide bridge-containing eukaryotic proteins after expression in prokaryotes; this task is solved with the subject of the present invention.

The subject of the invention is a process for  
35 the activation of heterologous, disulphide bridge-

containing eukaryotic proteins produced gene-technologically by expression in prokaryotes by

- a) digestion of the prokaryote cells,
- b) solubilisation of the eukaryotic proteins under  
5 denaturing and reducing conditions,
- c) separation of the reducing/denaturing agent,
- d) reactivation under oxidising conditions by
- e) conversion of the thiol groups of the solubilised  
10 proteins into the mixed disulphides of protein and  
glutathione by addition of GSSG under denaturing  
conditions,
- f) formation of active protein from the mixed  
disulphides at a GSH concentration of 0.5 to  
5 mmol/l, a pH value of 7 to 10.5 and in the  
15 presence of a non-denaturing concentration of a  
denaturing agent.

Preferred embodiments of this process are the subject of the subsidiary claims.

- As denaturing agent, there can, as a rule, be  
20 used a denaturing agent usually employed for activation  
under oxidising conditions or arginine; amongst the  
known denaturing agents, there is preferred guanidine  
hydrochloride or urea or its derivatives. Furthermore,  
arginine has proved to be suitable. Furthermore,  
25 mixtures of these denaturing agents can be used. This  
activation step is preferably also carried out in the  
presence of a foreign protein; as such, there is, as  
a rule, suitable any foreign protein, so long as it is  
not proteolytically active; bovine serum albumin (BSA)  
30 is preferably used, e.g. in an amount of 1 to 3 mg/ml.  
The addition of BSA brings about a slight increase of  
the yield and stabilisation of the protein (probably by  
protection against surface denaturing and/or proteo-  
lytic breakdown).

The other process conditions can correspond to the known and usual conditions for the reactivation steps from the prior art. The period of the activation (incubation) preferably amounts to 20 to 48 hours at room temperature. In the case of a longer incubation (48 hours) under reoxidation conditions, as a rule the stimulatability by CNBr-FCP decreases. The activation step is preferably carried out in the presence of EDTA, whereby the most expedient concentration amounts to about 1 mmol/l EDTA.

The process steps preceding and following the activation step (reoxidation/activation), such as cell digestion, solubilisation (solubilisation/reduction) and possibly one or more of the purification operations preceding and/or following the activation step, can be carried out according to known and usual methods for such processes from the prior art, e.g. from EP-A-0114506 and EP-A-0093619; however, for a result which is optimal with regard to yield and activation, it can be expedient to carry out individual or all process steps having regard to one or more of the here-described process embodiments. The expression is carried out in prokaryotes, preferably in *P. putida* and especially in *E. coli*. However, the process according to the invention is just as suitable when one expresses in other prokaryotes (e.g. *Bacilli*).

The cell digestion can be carried out by methods usual herefor, e.g. by means of ultrasonics, high pressure dispersion or lysozyme; it is preferably carried out in a buffer solution suitable for the adjustment of a neutral to weakly acidic pH value as suspension medium, such as e.g. in 0.1 mol/l tris-HCl. After the cell digestion, the insoluble components ("refractile bodies") are separated off in any desired way, preferably by centrifuging off at comparatively

high g values and comparatively long centrifuging times or by filtration. After washing with agents which do not disturb t-PA but possibly dissolve foreign proteins, e.g. water, phosphate buffer solution, possibly with addition of mild detergents, such as Triton, the precipitate (pellet) is subjected to the solubilisation (solubilisation/reduction). The solubilisation preferably takes place in the alkaline pH range, especially at  $\text{pH} = 8.6 \pm 0.4$  and in the presence of a reducing agent of the mercaptan group and of a denaturing agent.

As denaturing agent, there can be used the denaturing agents known and usual from the state of the art, e.g. from EP-A-0114506, and especially guanidine hydrochloride or urea. The concentration of guanidine hydrochloride expediently amounts to about 6 mol/l, that of urea to about 8 mol/l. Compounds of the general formula I can also be used.

As reducing agent from the mercapto group, there can be used e.g. reduced glutathione (GSH) or 2-mercaptoethanol, e.g. in a concentration of about 50 to 400 mmol/l and/or especially DTE (dithioerythritol) or DTT (dithiothreitol), e.g. in a concentration of about 80 to 400 mmol/l. The solubilisation expediently takes place at room temperature over a period (incubation) of 1 to several hours, preferably of two hours. For the prevention of the oxidation of the reducing agent by atmospheric oxygen, it can also be expedient to add EDTA. Besides the solubilising/reduction, the solubilising step also has a purification effect since a large part of the foreign proteins does not go into solution.

After the solubilisation, there takes place the separating off of the reduction and of the denaturing agents; a non-specific reoxidation can be prevented by

addition of a reducing agent (e.g. 2-mercaptoethanol) or by pH values 4.5 (cf. e.g. R. Rudolph, Biochem. Soc. Transactions, 13 (1985) 308 to 311). The separating off of the denaturing/reducing agents takes place  
5 by desalination over Sephadex G 25 in 0.01 mol/l HCl or 0.1 mol/l acetic acid. Alternatively, the separating off of the denaturing/reducing agents is possible by dialysis against the same solutions.

A further purification step can follow the  
10 reactivation step; as a rule, such a purification takes place by means of dialysis or also a subsequent isolation of the activated tPA, for example by affinity chromatography, for example over Lys-Sepharose.

The formation of the mixed disulphides of gene-  
15 technologically produced, heterologous, disulphide bridge-containing eukaryotic proteins with glutathione (in the following abbreviated to t-PASSG) simplifies not only the separation of foreign proteins in the denatured state but also the further purification of  
20 the native protein. A purification after modification of the thiol groups has the advantage that the protein is protected against air oxidation and thus is stable in a greater pH range and a change of the nett charging simplifies the purification. In particular, a separation  
25 from non-modified protein can advantageously be carried out by ion exchanger treatment.

For the formation of the mixed disulphides, the dialysed, reduced protein, purified from denaturing and reducing agents, is incubated with a diluted, e.g.  
30 0.2 mol/l, solution of GSSG containing a denaturing agent. The activation takes place after separation off of the denaturing and oxidation agent at a pH value of 7 to 10.5, at a GSH concentration of 0.5 to 5 mmol/l and with a non-denaturing concentration of the  
35 denaturing agent.

The pH optimum lies at 8.5, the yield is about twice as high as in the case of a reactivation with GSH/GSSG without separate formation of the mixed disulphides and the activated protein is stable in the regeneration buffer over a longer time.

According to the invention, it is possible so to activate t-PA from prokaryotes that there is achieved not only an activation of the normal biological activity but, in addition, a stimulatability in the above-defined sense is also achieved which far exceeds the stimulatability of the native t-PA and is greater than a factor of 10 and can even exceed a factor of 50.

A further eukaryotic protein which, according to the invention, can be activated after expression in prokaryotes is  $\beta$ -interferon.

The following Examples explain the invention in more detail without limiting it thereto. If not stated otherwise, statements of percentage refer to percent by weight and statements of temperature to degrees Celsius.

#### Example 1

##### a) Preparation of the "refractile bodies"

100 g E. coli moist cell mass, taken up in 1.5 l of 0.1 mol/l tris-HCl (pH 6.5) and 20 mmol/l of EDTA were homogenised (Ultra-Turrax, 10 sec.) and 0.25 mg/ml lysozyme added thereto. After 30 min. incubation at room temperature, it was again homogenised and cooled to 3°C. The cell digestion was achieved by high pressure dispersion (500 kg/cm<sup>2</sup>). Subsequently, it was after-rinsed with 300 ml of 0.1 mol/l tris/HCl (pH 6.5) and 20 mmol/l EDTA. After centrifuging (2 hrs. at 27000 g, 4°C), the pellet was taken up in 1.3 l of 0.1 mol/l tris/HCl (pH 6.5), 20 mmol/l EDTA and 2.5% Triton X-100 and homogenised. After renewed centrifuging (30 min. at 27000 g, 4°C), the pellet was taken up

in 1.3 l of 0.1 mol/l tris/HCl (pH 6.5), 20 mmol/l EDTA and 0.5% Triton X-100 and homogenised. Alternating centrifuging (30 min. at 27000 g, 4°C) and homogenisation of the pellets in 1 l of 0.1 mol/l Tris/HCl (pH 6.5) and 20 mmol/l EDTA was carried out a further three times.

The t-PA content of the "refractile bodies" preparations was quantified by SDS-PAGE, identification of the t-PA bands by "Western blotting" and densitometric analysis. In the case of SDS-PAGE and "Western blotting", the "refractile bodies" show a strong t-PA band with a molecular weight of about 60 kDa. The t-PA proportion of the total protein content of the "refractile bodies" amounts to about 21%.

b) Activation of t-PA via the mixed disulphides of t-PA and glutathione.

The "refractile bodies" used were obtained according to a). The reduction of the "refractile bodies" was carried out by 2 hours incubation at room temperature in 0.1 mol/l tris/HCl, pH 8.6, 1 mmol/l EDTA, 6 mol/l Gdn.HCl, 0.2 mol/l DTE at a protein concentration of about 1 mg/ml.

The reduced protein dialysed against 0.01 mol/l HCl was diluted in the ratio of 1:1 with 0.1 mol/l tris, pH 9.3, 9 mol/l urea and 0.2 mol/l GSSG and incubated for 5 hours at room temperature.

After acidification with conc. HCl to pH 3, there took place dialysis against 0.01 mol/l HCl at 4°C. After the dialysis, the total protein concentration amounted to 0.3 mg/ml. The optimum reactivation conditions were determined with the so-prepared t-PASSG.

c) pH optimum of the activation of t-PASSG

Here, as in the following optimising experiments, there was (1) used no GSSG and (2) the activation was

determined after 17 hours incubation at room temperature. Activation took place by a 1:100 dilution in 0.1 mol/l tris, 1 mmol/l EDTA, 0.5 mol/l L-arginine, 1 mg/ml BSA and 2 mmol/l GSH with variation of the pH value.

|    | pH   | yield (%) | stimulatability |
|----|------|-----------|-----------------|
| 10 | 6    | 0.04      | 3.3             |
|    | 6.5  | 0.37      | 9.5             |
|    | 7    | 1.35      | 11.4            |
|    | 7.5  | 5.66      | 7.1             |
|    | 8    | 7.32      | 8.2             |
|    | 8.5  | 8.65      | 7.0             |
|    | 9    | 8.59      | 8.7             |
|    | 9.5  | 8.32      | 11.7            |
| 15 | 10   | 6.15      | 12.5            |
|    | 10.5 | 3.07      | 11.2            |

The yield was determined in % of active t-PA, referred to the amount of protein used.

d) Reproducibility of the results of the activation of t-PASSG

In the case of identical activation conditions, in the case of different measurement series there are observed different yields which, inter alia, are caused by variations of the standard t-PA's. For the clarification of the breadth of error, all activation data after 1:100 or 1:200 dilution, respectively, in 0.1 mol/l tris/HCl, pH 8.5, 1 mmol/l EDTA, 0.5 mol/l L-arginine, 1 mg/ml BSA and 2 mmol/l GSH, are summarised.

|               | experiment | yield (%)   | stimulatability |
|---------------|------------|-------------|-----------------|
| 5             | 1          | 8.65        | 7.0             |
|               | 2          | 4.47        | 9.3             |
|               | 3          | 4.49        | 9.7             |
|               | 4          | 8.50        | 6.5             |
|               | 5          | 3.45        | 17.2            |
|               | 6          | 4.32        | 8.3             |
|               | 7          | 3.29        | 14.0            |
|               | 8          | 3.54        | 13.4            |
| 10            | 9          | 5.07        | 16.4            |
| average value |            | 5.1 +/- 1.9 | 11.3 +/- 3.8    |

e) Stability of the activated protein

Activation took place in the said Example by a  
 15 1:200 dilution in 0.1 mol/l tris/HCl, 1 mmol/l EDTA,  
 0.5 mol/l L-arginine, 1 mg/ml BSA and 2 mmol/l GSH.

|    | time<br>(h) | yield (%)<br>pH 9.5 | stim. |
|----|-------------|---------------------|-------|
| 20 | 1           | 0                   | -     |
|    | 6           | 0.89                | 15.5  |
|    | 23          | 2.43                | 23.1  |
|    | 47          | 2.83                | 23.6  |
|    | 71          | 2.62                | 21.5  |
|    | 215         | 2.21                | 22.6  |
| 25 | 239         | 2.28                | 14.3  |



Patent Claims

1. Process for the activation of heterologous, disulphide bridge-containing eukaryotic proteins produced gene-technologically by expression in prokaryotes by
  - a) digestion of the prokaryote cells,
  - b) solubilisation of the eukaryotic proteins under denaturing and reducing conditions,
  - c) separation of the reducing/denaturing agents,
  - d) reactivation under oxidising conditions by
  - e) conversion of the thiol groups of the solubilised proteins into the mixed disulphides of protein and glutathione by addition of GSSG under denaturing conditions,
  - f) formation of active protein from the mixed disulphides at a GSH concentration of 0.5 to 5 mmol/l, a pH value of 7 to 10.5 and in the presence of a non-denaturing concentration of a denaturing agent.
2. Process according to claim 1, characterised in that one carries out the expression in *E. coli* or *P. putida*.
3. Process according to claim 1 or 2, characterised in that, in the reactivation step, as denaturing agent, one uses arginine, guanidine hydrochloride and/or at least one compound of the general formula  $R_2\text{-CO-NRR}_1$  (I), in which R and  $R_1$  signify H or alkyl with 4 C-atoms and  $R_2$  signifies H or  $\text{NHR}_1$  or alkyl with 1 to 3 C-atoms.
4. Process according to claim 3, characterised in that the concentration of arginine and/or guanidine hydrochloride amounts to 0.1 to 1.0 mol/l, especially 0.25 to 0.8 mol/l.
5. Process according to claim 3, characterised in that the concentration of the compound of the general



-15-

formula I amounts to 0.5 to 4 mol/l, especially 1 to 3.5 mol/l.

6. Process according to one of the preceding claims, characterised in that, in the step of reactivation,  
5 one works in the presence of a non-proteolytically effective protein, especially in the presence of bovine serum albumin.

7. Process according to one of the preceding claims, characterised in that one carries out the cell  
10 digestion by means of ultrasonics, high pressure dispersion or lysozyme.

8. Process according to claim 7, characterised in that one carries out the digestion in a dilute aqueous buffer solution, especially in 0.1 mol/l tris, at a  
15 neutral to weakly acidic pH value.

9. Process according to one of the preceding claims, characterised in that, after the cell digestion, one separates off the insoluble components.

10. Process according to one of the preceding claims, characterised in that, in the step of solubilisation,  
20 one works at an alkaline pH value in the presence of a reducing agent of the mercapto group and in the presence of a denaturing agent.

11. Process according to claim 10, characterised in  
25 that one works in the presence of guanidine hydrochloride and/or of the general formula I as denaturing agent.

12. Process according to claim 11, characterised in that the concentration of guanidine hydrochloride  
30 amounts to 6 mol/l, that of the compound of the general formula I to 8 mol/l.

13. Process according to one of claims 10 to 12, characterised that one works in the presence of DTE,  $\beta$ -mercaptoethanol, cysteine or GSH.



-16-

14. Process according to one of the preceding claims, characterised in that one carries out purification and separation of reducing, oxidising and denaturing agents by means of steric exclusion chromatography or  
5 dialysis.
15. Process according to one of the preceding claims, characterised in that, after the step of reactivation, one carries out a purification step by means of dialysis.
- 10 16. Process according to one of claims 1 to 15, characterised in that one uses t-PA as gene-technologically produced eukaryotic protein.
- 15 17. Stimulatable, non-glycosylated t-PA, obtainable according to the process according to one of claims 1 to 16.
18. Process according to claim 6, characterised in that one separates the mixed disulphide of protein and glutathione from the non-modified protein by ion exchanger treatment.