IN THE MATTER OF European Patent No. 0 393 725 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. 0 393 725.

Dated this 24th day of July, 1995

W. G. M. Shipley

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

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 host cells. It is assumed that the formation of such "refractile bodies" is a result of the high protein concentrations 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 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.

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) 187-198; R. Rudolph et al., Biochemistry 18 (1979) 5572-5575).

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 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 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 the use of guanidine hydrochloride, it is thereby diluted to values below 0.5 mole/l. In the case of enzymes with free SH groups, the addition of agents protecting the SH groups proves to be advantageous (cf. e.g. R. Jaenicke, Journal Polymer Science, Part C, 16 (1967) 2143-2160).

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 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-sulphonate groups are converted into -S-S- group 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 typical example in which the above-discussed problems arises is t-PA.

The main component of the protein matrix of coagulated blood is polymeric fibrin. This protein 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 a 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) 2912-2019; Nieuwenhiuzen et al., Biochemica et Biophysica Acta 755 (1983) 531-533). 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 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) 775-780.

According to EP-A-0093639, for the reactivation of t-PA, the cell pellets obtained from E. coli are suspended in 6 mole/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 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. Vereijen, Thromb. Haemostas., 48 (3), 260-269 (1982).

Orsini and Goldberg (J. Biol. Chem. 253 (1978) 3453-3458) describe the renaturing of reduced chymotripsinogen A by conversion of the denatured protein in a renaturing buffer which contains GSH, GSSG and non-denaturing concentrations of guanidine-HCl or urea.

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; it contains a free thiol group and 17 SS bridges which theoretically can be linked in 2.2×10^{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 to a proteolytically active t-PA but which shows no measurable stimulatability; an activation process which leads to stimulatable t-PA is not known.

Therefore, it is the task of the present invention to make available a process for the complete 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 the activation of gene-technologically produced, heterologous, disulphide bridge-containing eukaryotic proteins after expression in prokaryotes by cell digestion, solubilisation under denaturing and reducing conditions

and activation under oxidising conditions in the presence of GSG/GSSG, which is characterised in that, in the step of the activation, one works at a pH value of 8 to 12, a GSH concentration of 0.1 to 20 mmol/l, a GSSG concentration of 0.01 to 3 mmol/l and with a non-denaturing concentration of the denaturing agent and that, in the reactivation step, as denaturing step, there is used arginine and/or at least one compound of the general formula R_2-CO-NRR_1 (I) as denaturing agent, whereby R and R_1 signify H or alkyl with 1 to 4 C-atoms and R_2 signifies H or NHR_1 or alkyl with 1 to 3 C-atoms, with the proviso that R and R_1 are not simultaneously H.

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

As denaturing agent, there are used arginine or urea derivatives according to formula (I). Furthermore, 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) 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 proteolytic 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. The half life time of the activation lies, in the presence of 0.5 mmol/l reduced (GSH) and oxidised (GSSG) glutathione, at about 10 to 15 hours at 20°C. 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 known 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. In particular, it is also possible to carry out the step of activation according to the invention in the mixture obtained after digestion without previous denaturing and/or reduction but with lower yield. 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 the washing with agents which do not disturb t-PA but possibly dissolve foreign proteins, e.g. water, phosphate buffer solution, possibly with the 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 pH = 8.6 ± 0.4 and in the presence of a reducing agent of the mercaptan group and of a denaturing agent.

As denaturing agent, for the solubilisation 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 be expedient to add EDTA. Besides the solubilising/reduction, the solubilising step also has a purification effect since a large part of material (foreign proteins) not cross-reacting immunologically with t-PA goes into solution.

After the solubilisation and before the activation step, per se known and usual purification steps can be introduced; as purification methods, there come into

question, e.g. steric exclusion chromatography (SEC) (in the presence of guanidine hydrochloride or urea) or ion exchangers (in the presence of urea or its derivatives); a non-specific reoxidation can be prevented by addition of a reducing agent (e.g. 2-mercaptoethanol) or by pH value 4.5 (cf. e.g. R. Rudolph, Biochem. Soc. Transactions, 13 (1985) 308-311). If DTE were used in the preceding solubilisation step, this must be separated off in a purification step. The purification can take place e.g. by SEC over Sephadex G 100 in the presence of guanidine hydrochloride and of a reducing agent, e.g. of GSH, at a pH of 1 to 4 (in this step, a large amount of foreign protein can be separated off); or by separating off of the denaturing/reducing agent 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 agent is possible by dialysis against the same solutions.

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

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 factor 10 and can even exceed factor 50.

A further eukaryotic protein which, according to the invention, can be activated after expression in prokaryotes is β-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 temperatures 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^2). 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. Alternatingly 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 further carried out three times.

The t-PA content of the "refractile bodies" preparations was quantified by the SDS-PAGE, identification of the t-PA bands by "Western blotting" and densitomeric analysis. In the case of the 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) Solubilising/reduction of the "refractile bodies"

"Refractile bodies" were incubated at a protein concentration of 1 to 5 mg/ml in 0.1 mol/l of tris/HCl (pH 8.6), 6 mol/l guanidine hydrochloride, 0.15 to 0.4 mol/l DTE and 1 mmol/l EDTA for 2 to 3 hrs. at room temperature. Thereafter, insoluble material (cell wall fragments etc.) was centrifuged off (e.g. 30 min. at 35000 to 50000 g, 4°C). The pH of the supernatant was adjusted with conc. HCl to pH 3. Denaturing and reducing agents were separated off by dialysis against 0.01 mol/l HCl at 4°C.

c) Reoxidation/activation

Reoxidation/activation took place by means of a 1:50 to 1:200 dilution in 0.1 mol/l tris/HCl (pH 10.5), 1 mmol/l EDTA, 1 mg/ml BSA, 0.5 mol/l L-arginine, 2 mmol/l GSH, 0.2 mmol/l GSSG. After 17 to 24 hours activation at about 30°C, there was determined the activity and the yield in comparison with the activity of native glycosilated t-PA from eukaryonts.

Yield referred to the total protein content of the "refractile bodies": 2.5 +/- 0.5%
stimulatability: 10 ± 5.

Yield referred to the t-PA portion of the "refractile bodies": about 12%.

d) Reoxidation/activation without separation of the denaturing/reducing agent

"Refractile bodies" were incubated at room temperature at a protein concentration of 1.25 mg/ml in 0.1 mol/l tris/HCl (pH 8.6), 6 mol/l guanidine hydrochloride, 0.2 mol/l DTE and 1 mmol/l EDTA for 2 hours at room temperature. Thereafter, reoxidation was immediately initiated by a 1:100 dilution in 0.1 mol/l tris/HCl (pH 10.5), 1 mmol/l EDTA, 1 mg/ml BSA, 0.3 mol/l L-arginine and the amounts of GSSG

given in the Table. In addition, in the activation batch was present a residual concentration of 0.06 mol/l guanidine hydrochloride and 2 mmol/l DTE.

Dependency of the activation yield upon the GSSG concentration in the case of activation without separation of the denaturing/reducing agent

GSSG (mmol/l)	yield' (%)	stimulatability (factor
0.2	0	-
1	0.13	4.0
5	1.49	7.4
6	1.28	5.4
7	1.04	5.8
9	0.98	5.2
10	1.77	10.0
15	0	-
20	0	-

' = yield of active t-PA, referred to the total protein content of the "refractile bodies".

Example 2

An RB ("refractile bodies") preparation (about 5 mg) was incubated for 2 to 3 hours at room temperature in 1 ml of 0.1 mol/l tris/HCl (pH = 8.6), 6 mol/l guanidine hydrochloride and 0.15 - 0.2 mol/l DTE. Insoluble material (cell wall fragments etc.) were thereafter separated off by centrifuging (20 minutes at 17000 g). Denaturing and reducing agents were removed by gel filtration over Sephadex G 25 (superfine) in 0.01 mol/l HCl. The sample was thereby diluted by about the factor 5 to 10. The reduced material was stored at -20°C in 0.01 mol/l HCl.

Example 3

In the following Tables is summarised the influence of various parameters according to the

invention on the activation and stimulatability of t-PA. For these reoxidation experiments, the reduced protein solubilised according to Example 2 was not further prepurified.

The reduced protein (in 0.01 mol/l HCl) was activated by dilution of 1:10 to 1:500 in "reoxidation buffer". The activation was determined at room temperature after 22 to 48 hours incubation. The activity of the reoxidised protein refers to a "standard reoxidation" (= 100%) in:

0.1 mol/l tris/HCl (pH = 10.5) + 1 mmol/l EDTA
+ 0.5 mol/l L-arginine
+ 1 mg/ml BSA
+ 0.5 mmol/l GSH (reduced glutathione)
+ 0.5 mmol/l GSSG (glutathione disulphide).

The stimulatability is calculated from

$$\Delta E_{+\ CNBr\ FCP} \ / \ \Delta E_{-CNBr\ FCP}$$

(cf. W. Nieuwenhuizen et al., Biochemica et Biophysica Acta 755 (1983) 531-533). The activity (in percent) and the stimulatability (factor) was determined according to J.H. Verheijen, Thromb. Haemostas. 48(3), 266-269, (1982).

The following results were obtained:

1. Dependency of the activation yield upon the addition of L-arginine or guanidine hydrochloride.

   Reoxidation in 0.1 mol/l tris/HCl (pH 10.5)
   + 1 mmol/l EDTA
   + 1 mg/ml BSA
   + 0.5 mmol/l GSH
   + 0.5 mmol/l GSSG

a) L-arginine

L-arginine (mol/l)	activity (%)	stimulatability (factor)
0	4	2.5
0.25	98	7.5
0.5	100	21.9
0.75	27	16.3
1.0	23	3.5

In the case of this experiment, it is to be borne in mind that t-PA is inhibited by L-arginine. The decrease of the activity yield at higher L-arginine concentrations is, therefore, to be corrected with regard to the inhibition.

b) Guanidine hydrochloride (Gdn/HCl)

Gdn/Hcl (mol/l)	activity (%)
0	11
0.25	22
0.5	53
0.75	58
1.0	12

2. Dependency of the activation yield upon addition of urea and urea derivatives.

Reoxidation in 0.1 mol/l tris (pH 10.5), 1 mmol/l EDTA, 1 mg/ml BSA, 5 mmol/l GSH, 0.2 mmol/l GSSG

a) Urea

urea (mol/l)	activity (%)
0	1
0.5	20
1	59
1.5	126
2	162
2.5	141
3	72
4	12
5	0

b) Methylurea

methylurea (mol/l)	activity (%)
0.5	22
1	174
1.5	313
2	375
2.5	332
3	215
4	12
5	0

c) Ethylurea

ethylurea (mol/l)	activity (%)
0.5	46
1	212
1.5	323
2	300
2.5	107
3	19
4	0
5	0

d) Dimethylurea

dimethylurea (mol/l)	activity (%)	stimulatability (factor)
0.5	167	8.8
1	256	8.9
1.5	283	9.4
2	177	7.7
2.5	78	8.9
3	23	9.9
4	4	8.6
5	2	3.5

3. Dependency of the activation yield upon the addition of fatty acid amides:

Reoxidation in 0.1 mol/l tris (pH 10.5), 1 mmol/l EDTA, 1 mg/ml BSA, 5 mmol/l GSH, 0.2 mmol/l GSSG.

a) Formamide

formamide (mol/l)	activity (%)
0	42
0.5	59
1	175
1.5	245
2	325
2.5	423
3	444
4	416
5	341

b) Methylformamide

methylformamide (mol/l)	activity (%)
0.5	100
1	135
1.5	304
2	389
2.5	466
3	452
4	425
5	121

c) Acetamide

acetamide (mol/l)	activity (%)
0.5	72
1	134
1.5	207
2	261
2.5	204
3	237
4	198
5	141

d) Propionamide

propionamide (mol/l)	activity (%)
0.5	95
1	99
1.5	197
2	150
2.5	101
3	39
4	2
5	0

e) Butyramide

butyramide (mol/l)	activity (%)
0.5	55
1	52
1.5	17
2	0

4. Dependency of the activation yield on the pH value

Reoxidation in 0.1 mol/l tris/HCl + 1 mmol/l EDTA
+ 0.5 mol/l L-arginine
+ 1 mg/ml BSA
+ 0.5 mmol/l GSH
+ 0.5 mmol/l GSSG

pH	activity (%)	stimulatability (factor)
7	1	-
8	22	3.0
9	89	13.6
10	105	20.3
11	95	21.3

5. Dependency of the activation yield upon the GSH/GSSG concentration

Reoxidation in 0.1 mol/l tris/HCl, pH 10.5
+ 1 mmol/l EDTA
+ 0.5 mol/l L-arginine
+ 1 mg/ml BSA

a) + 1 mmol/l GSH

GSSG (mmol/l)	activity (%)	stimulatability (factor)
0.1	239	14.9
0.2	273	15.3
0.5	193	13.3
1	198	12.5
5	17	2.1
10	0	-
20	0	-

b) + 0.2 mmol/l GSSG

GSH (mmol/l)	activity (%)	stimulatability (factor)
0.05	15	2.2
0.1	40	3.8
0.2	112	6.8
0.5	142	7.4
1	273	6.8
5	260	7.9
10	143	6.3
20	55	5.1

6. Dependency of the activation yield upon the protein concentration in the case of the reoxidation (dilution 1:20 - 1:500)

Reoxidation on 0.1 mol/l tris/HCl (pH 10.5)
+ 1 mmol/l EDTA
+ 0.5 mol/l L-arginine
+ 1 mg/ml BSA
+ 0.5 mmol/l GSH
+ 0.5 mmol/l GSSG

dilution	activity (%)	stimulatability (factor)
1:10	29	15.3
1:20	45	25.4
1:50	69	37.9
1:100	100	37.9
1:200	79	52.7
1:500	29	28.7.

7. Dependency of the activation yield upon the addition of BSA

Reoxidation in 0.1 mol/l tris/HCl (pH 10.5)
+ 1 mmol/l EDTA
+ 0.5 mol/l L-arginine
+ 0.5 mmol/l GSH
+ 0.5 mmol/l GSSG

BSA (mg/ml)	activity (%)
0	47
0.5	83
1	100
3	102
5	52

The Figures 1 and 2 show the activity with and without CNBr-FCP in the standard test after 17 hours reoxidation at room temperature in 0.1 mol/l tris/HCl (pH = 10.5) + 1 mmol/l EDTA + 0.5 mol/l L-arginine + 1 mg/ml BSA + 0.5 mmol/l GSH + 0.5 mmol/l GSSG. In the Figs. 1 and 2, the curves (A) signify the activity in the presence of CNBr-FCP, the curves B the activity without CNBr-FCP.

Example 4

Activation of gene-technologically produced interferon-β

"Refractile bodies" were produced according to the previously mentioned methods. The reduction/solubilisation of the "refractile bodies was carried out as follows: The pellet was incubated for 3 hours at 25°C in 10 ml 9.1 mol/l tris/HCl, pH 8.6, 6 mol/l Gdn.HCl, 1 mmol/l EDTA and 0.2 mol/l DTE and, after 30 minutes centrifuging at 4°C and 48000 g, the pH of the supernatant was adjusted to about 3 with concentrated HCl. Subsequently, a gel filtration over Sephadex G25F in 0.01 mol/l HCl was carried out.

The eluate was investigated for conductivity, protein concentration and reactivatability.

The activity of the reoxidised protein refers to a "standard activation" (= 100%) in 0.1 mol/l tris/HCl, pH 10.5, 1 mmol/l EDTA, 5 mmol/l GSH, 0.5 mmol/l GSSG and 0.25 mol/l L-arginine.

a) Dependency of the activation yield on the addition of L-arginine

The eluate was diluted 1:50 with 0.1 mol/l tris/HCl, pH 8.5, 1 mmol/l EDTA, 5 mmol/l GSH, 0.5 mmol/l GSSG and activated for 20 hours at 0°C.

L-Arginine dependency of the activation

L-arginine (mol/l)	activity (%)
0	8
0.25	8
0.5	15
0.75	15

b) Dependency of the activation yield on the addition of urea

The activation solution corresponded to that of point a) but it was activated for 17 hours at 0°C.

Urea dependency of the activation

urea (mol/l)	activity (%)
0	13
0.5	100
1	200
1.5	100

c) Dependency of the activation yield upon the addition of formamide

Activation as in a); the samples were investigated after 17 hours activation at 0°C.

Formamide dependency of the activation

formamide (mol/l)	activity
0	13
1	13
2	13
3	0
4	0

d) Dependency of the activation yield on the redox buffer

The eluate was diluted 1:50 in 0.1 mol/l tris/HCl, pH 8.5, 1 mmol/l EDTA and 0.25 mol/l L-arginine and the samples investigated after 17 hours activation at 0°C.

GSH/GSSG dependency of the activation

GSH (mmol/l)	GSSG (mmol/l)	activity (%)
1	0.5	6
5	0.5	13
10	0.5	25
20	0.5	25
5	0.1	13
5	0.5	13
5	1.0	13
5	5	6

e) Dependency of the activation yield on the addition of BSA

The eluate was diluted 1:50 in 0.1 mol/l tris/HCl, pH 8.5, 1 mmol/l EDTA, 5 mmol/l GSH, 0.5 mmol/l GSSG and 0.25 mol/l L-arginine and investigated after 17 hours activation at 0°C.

BSA dependency of the activation

BSA (mg/ml)	activity (%)
0	13
1	13
2	25
5	13

f) Dependency of the activation yield on the pH

The eluate was diluted 1:50 in 0.1 mol/l tris/HCl, 1 mmol/l EDTA, 5 mmol/l GSH, 0.5 mmol/l GSSG and 0.25 mol/l L-arginine and investigated after 17 hours activation at 0°C.

pH dependency of the activation

pH	activity (%)
6.5	0
7.5	6
8.5	13
9.5	50
10.5	100

Patent Claims

1. Process for the activation of gene-technologically produced, heterologous, disulphide bridge-containing eukaryotic proteins after expression in prokaryotes by cell digestion, solubilisation under denaturing and reducing conditions and reactivation under oxidising conditions in the presence of GSH/GSSG, characterised in that, in the step of the reactivation, one works at a pH value of 8 to 12, a GSH concentration of 0.1 to 20 mmol/l, a GSSG concentration of 0.01 to 3 mmol/l and with a non-denaturing concentration of the denaturing agent and that, in the reactivation step, as denaturing agent, one uses arginine and/or at least one compound of the general formula R_2-CO-NRR_1 (I), whereby R and R_1 signify H or alkyl with 1 to 4 C-atoms and R_2 signifies H or NHR_1 or alkyl with 1 to 3 C-atoms, with the proviso that R and R_1 are not simultaneously H.

2. Process according to claim 1, characterised in that, in the reactivation step, the pH value amounts to 9.5 to 11.

3. Process according to one of claims 1 and 2, characterised in that, in the reactivation step, the GSH concentration amounts to 0.2 to 10 mmol/l and/or the GSSG concentration to 0.05 to 1 mmol/l.

4. Process according to one of the preceding claims, characterised in that one carries out a purification step after the solubilisation and before the reactivation.

5. Process according to one of claims 1 to 3, characterised in that the reactivation is carried out without previous separation of the denaturing/reducing agent, whereby the reaction solution is diluted after denaturing/reducing with reactivation buffer and, in the following reactivation, the GSSG concentration exceeds the remaining residual concentration of DTE.

6. Process according to one of claims 1 to 5, characterised in that one carries out the expression in E. coli or P. putida.

7. Process according to one of claims 1 - 6, characterised in that the concentration of arginine amounts to 0.1 to 1.0 mol/l, especially 0.25 to 0.8 mol/l.

8. Process according to one of claims 1 - 6, characterised in that the concentration of the compound of the general formula I amounts to 0.5 to 4 mol/l, especially 1 to 3.5 mol/l.

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

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

11. Process according to claim 10, characterised in that one carries out the digestion in a dilute aqueous buffer solution, especially in 0.1 mol/l tris, at a neutral to weakly acidic pH value.

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

13. Process according to one of the preceding claims, characterised in that, in the step of solubilisation, 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.

14. Process according to claim 13, characterised in that one works in the presence of a compound of the

general formula I as denaturing agent.

15. Process according to claim 14, characterised in that the concentration of the compound of the general formula I amounts to 8 mol/l.

16. Process according to one of claims 13 to 15, characterised in that one works in the presence of DTE, β-mercaptoethanol, cysteine or GSH.

17. 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 dialysis.

18. 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.

19. Process according to one of claims 1 to 18, characterised in that one uses t-PA as gene-technologically produced eukaryotic protein.

20. Process according to one of claims 1 to 18, characterised in that one uses interferon β as gene-technologically produced eukaryotic protein.