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Veröffentlicht:
— *Mit internationalem Recherchenbericht.*
— *Vor Ablauf der für Änderungen der Ansprüche geltenden Frist: Veröffentlichung wird wiederholt, falls Änderungen eintreffen.*

Zur Erklärung der Zweibuchstaben-Codes, und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

(54) Title: METHOD FOR STABILISING PROTEINS IN COMPLEX MIXTURES DURING THEIR STORAGE IN AQUEOUS SOLVENTS

(54) Bezeichnung: VERFAHREN ZUR STABILISIERUNG VON PROTEINEN IN KOMPLEXEN MISCHUNGEN BEI DEREN LAGERUNG IN WÄSSRIGEN LÖSUNGSMITTELN

(57) Abstract: The invention relates to a method for storing proteins in an aqueous solvent. The addition of cysteine delays the decline of the active concentration of the protein. The method is suitable for use in the production of proteins in micro-organisms.

(57) Zusammenfassung: Die vorliegende Erfindung betrifft ein Verfahren zur Lagerung von Proteinen in einem wäßrigen Lösungsmittel. Die Zugabe von Cystein verzögert die Abnahme der wirksamen Konzentration des Proteins. Das Verfahren eignet sich zur Anwendung bei der Produktion von Proteinen in Mikroorganismen.

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Description

Process for the stabilization of proteins in complex mixtures during their storage in aqueous solvents

The present invention relates to a process for the storage of proteins in an aqueous solvent.

The chemical state of the SH groups of proteins or other oxidation-sensitive structures often has an effect on the identity, activity or effective concentration of proteins. Identity means the respective folding of a protein. Activity should be understood as meaning enzyme activity. The effective concentration of a protein should be the proportion of the protein in a solution which is correctly folded with respect to the biological in vivo function.

It is known from the literature that structure-modifying oxidations of proteins can be suppressed by the use of thiol reagents such as, for example, 2-mercaptoethanol or cysteine.

For example, D-amino acid oxidase (DAO) can be stabilized by thiols. The flavoprotein DAO catalyzes the stereospecific deamination of D-amino acids to the corresponding α-ketoacids and ammonium (P. Golini et al. Enzyme and Microbial Technology, 17, 1995, 324 – 329). However, the addition of thiols such as, for example, cysteine can also reduce the activity of proteins. This effect can also be explained by the presence of cysteine radicals. An example of this is aminoacylase. Aminoacylase is a dimeric enzyme having one Zn^{2+} atom per subunit. Each subunit of the enzyme contains 2 cysteine SH groups and 2 disulfide bonds. The chemical modification of the SH groups such as the breaking of the disulfide bonds can lead to an inactivation of the enzyme. It was possible to show that by the addition of 2-mercaptoethanol the activity of the aminoacylase is reduced, whereas after removal of the 2-mercaptoethanol by dialysis or gel filtration the original enzyme activity can be almost completely restored (W. Kördel and F. Schneider, Biochem. Biophys. Acta 445, 1976, 446-457).

For cysteine and some derivatives, it was possible for certain preparation forms and specific applications to demonstrate an antibacterial, antiviral or antifungal activity to a certain extent. Thus, for example, it was possible to show that the addition of cysteine is suitable to a certain extent as protection against the spoilage of foods (US 4937085 A).

With the aid of genetic engineering processes, it is possible to synthesize recombinant proteins such as insulin or its precursors, and also insulin derivatives which have amino acid compositions differing from the derived gene sequence (e.g. human), in genetically modified microorganisms such as the bacterium Escherichia coli.

Recombinant synthesis in microorganisms is carried out with the aid of expression vectors. These expression vectors consist of a vector plasmid, which should contain a control sequence for the replication of the plasmid as well as a selection gene (antibiotic resistance gene, metabolic marker, inter alia) into which the coding region of the gene for the protein of interest (e.g. insulin) was inserted under the control of a promoter which is active in the chosen microorganism (for E. coli, for example, the lac promoter).

A process for the production of recombinant proteins (e.g. insulin; insulin derivatives, inter alia) with the co-operation of genetically modified microorganisms is composed of a series of process steps which intermesh with each other and must be co-ordinated with one another.

Thus, for example, a process for the production of human insulin in E. coli can be constructed from the following process steps.

Fermentation of the microorganisms – cell separation – cell disruption – isolation and intermediate storage of the fusion protein with cysteine refolding into the native spatial structure with revelation of the disulfide bridges with subsequent separation of foreign proteins not containing material of value – enzymatic cleavage to the arginylinsulin – basic purification of the aqueous protein solution – 1st chromatographic purification – enzymatic cleavage to human insulin – 2nd chromatographic purification – high purification by means of HPLC – recrystallization – drying.

The large number of individual measures carried out as a rule leads to considerable losses in the total yield, as in each step losses due to the specific yield of the chosen process step are unavoidable.

By optimization of intermediate steps, the total yield can be improved. There is a considerable interest in such processes in order that a better economical utilization of the resources employed and a decrease in environmental pollution is made possible.

For example, EP 0906918 describes an improved process for the production of a precursor of insulin or insulin derivatives having correctly linked cystine bridges in the presence of cysteine or cysteine hydrochloride and of a chaotropic auxiliary.

Insulin derivatives are derivatives of naturally occurring insulins, namely human insulin or animal insulins. These insulin derivatives differ from the otherwise identical naturally occurring insulin by the absence and/or the replacement of at least one amino acid radical occurring in the naturally occurring insulin concerned by another genetically encodable amino acid residue and/or the addition of at least one genetically encodable amino acid residue.

During the storage of proteins, a decrease in the effective concentrations usually occurs.

The storage of production products between individual process steps can be necessary for various reasons. It mat thus be the case, for example, that the processing capacity of a subsequent further industrial processing step is not adequate in order to accept the total amount of product of the preceding process step.

The time needed for intermediate storage may be of differing length. Owing to capacity, the need to co-ordinate industrial units, the required delivery of more chemicals or appliances or for other reasons, the intermediate storage necessary may extend to several weeks.

An effective way of increasing the yield is opened up when the losses of active protein occurring during the unavoidable intermediate storage of

products from individual process steps before their further processing can be kept within limits which are as tight as possible.

Not described until now is the use of cysteine or derivatives for the control of yield losses of active protein during the intermediate storage of production products of differing composition and form of state from individual process steps of industrial production processes including biological components, for example with participation of enzyme catalysts or genetically modified microorganisms. Such processes are used, for example, in the preparation of insulin.

Depending on the process step, the production products to be intermediately stored can consist of different amounts of complex macromolecules of of biological nature (proteins, DNA, fats), microorganisms, buffer substances and starting materials, inter alia.

The production process as a rule aims at preparation of a substance which is as uniform as possible, in the case of insulin production to insulin. If production products have to be intermediately stored, in the case of the preparation of a protein, such as, for example, insulin, loss of effective concentration of this protein regularly occurs.

Storage of a protein should be understood as meaning any storage of the protein, regardless of the volumetric amount in which the protein is present and the time period over which the storage is carried out, or the temperature conditions under which it takes place. The storage of proteins normally takes place in aqueous solutions.

In addition to constituents of nutrient media for the culture of microorganisms in defined form or as complete media containing, in particular, carbon sources or nitrogen sources, amino acids and inorganic salts and trace elements, also buffer components of different chemical buffer types, as well as macromolecules of biological origin such as DNA or fats, an aqueous solution of a protein may additionally contain organic or inorganic compounds such as, for example, Na dodecylsulfate or K acetate and also proportions of solvents of differing priority, such as methanol or petroleum ether.

The present invention relates to a process for the storage of a protein in an aqueous solvent, which comprises delaying the temporal decrease in the effective concentration of the protein by addition of cysteine to the aqueous solvent.

The process is suitable for the stabilization of [lacuna] slowing of the temporal decrease in the effective concentration of a protein during its storage in aqueous solutions. The process can be used, for example, in the production of proteins by microorganisms. Examples of appropriate microorganisms are, inter alia, especially bacteria, in this case in particular Escherichia coli, yeasts, such as, especially, Saccharomyces cerevisiae or Pichia pastoris, and also insect cells. In the preferred embodiment, these microorganisms can be transformed using expression vector constructs for the induced or constitutive expression of said protein. The microorganisms are cultured and disrupted after synthesis of the protein. Suitable disruption methods are principally all processes which are suitable for the release of the protein from the bacteria. Disruption processes which are applicable are, for example, ultrasound, chemical methods using K acetate, Na dodecylsulfate or lysozyme, heating of the microorganisms or the French press. The disruption of the microorganisms may be omitted if the protein to be prepared is secreted directly into the medium. In principle, the process is also applicable to extruded proteins.

The complex protein mixtures which result when the microorganisms are disrupted or extrude protein directly into the medium are usually in aqueous solution. The proteins may be dissolved therein or present in suspended form. These protein solutions are preferably stored between 0°C and 50°C or between 5°C and 30°C or at 5°C. To delay the inactivation, cysteine is added to this protein mixture. The concentration of cysteine in the protein mixture may be between 100 and 500 mM or 150 and 220 mM. 170 mM are preferably added. In this way, proteins in complex molecule solutions can be stored for up to several weeks with only a slight decrease in the effective protein concentration. The process is applicable to the synthesis of heterologous proteins, in particular in microorganisms, after their disruption with subsequent purification and possible renaturation, preferably for the preparation of insulin and its precursor.

Proteins used for storage in a process of the invention are preferably insulin or insulin derivatives.

The present invention further relates to a process for the preparation of a heterologous protein, comprising the expression of the heterologous protein or its precursor in a transformed microorganism, which heterologous protein is then stored by means of a process according to the present invention. This heterologous protein or a precursor thereof is optionally then renatured, purified of leader sequences or otherwise processed and finally prepared to give the desired product after purification and isolation. Suitable heterologous proteins are preferably insulin or insulin derivatives and precursors of insulin.

Examples:

During the implementation of the processes for the preparation of insulin, it was found that by the addition of cysteine to the fusion protein the product remains stable on storage for months. In comparison, the untreated product loses some of its activity irreversibly after just a few days.

The precursor molecules of human insulin and of an insulin derivative are used in the following examples. The structure and amino acid sequence are disclosed in EP 906 918 by sequence protocols. The precursor of human insulin has the sequence of naturally occurring human insulin. The precursor of the insulin derivative contains a glycine instead of an arginine in position 21 of the A chain and two arginine molecules in positions 31 and 32 at the C-terminal end of the B chain.

Fusion proteins of the insulins can be prepared by fermentation of genetically modified E. coli cells according to EP 0 489 780 and EP 0 906 918. A protein suspension is obtained here which contains approximately 20 to 25% dry matter and 40 to 50% of foldable insulin.

For the sought-after stabilization of the protein by means of cysteine, 75 kg of cysteine hydrochloride × H_2O are introduced into about 2500 kg of this protein suspension (corresponding to one fermentation batch) in the course of 20 minutes with vigorous stirring. The pH falls from 7.0 to 2.5 in the course of this. The suspension becomes very pasty in the pH range around 5 to become readily stirrable again from around pH 4. Under these experimental conditions, a cysteine hydrochloride concentration of 170 mM is established in the protein suspension. The protein suspension is

subsequently stirred for 60 min, after which it can remain without further stirring until working-up.

Example 1:

For experimental confirmation of the preserving action of cysteine, the following laboratory experiments were carried out:

Fusion protein of human insulin and fusion protein of the insulin derivative prepared according to EP 906 918 were stored with and without cysteine at 5°C and at room temperature for up to 2 months. During the experiment, aliquots were removed from the batches. The fusion protein was converted into the prepro form of human insulin or prepro form of the insulin derivative by reductive folding (EP 906 918). The amount of cysteine necessary for folding was added to batches which were stored without cysteine hydrochloride monohydrate about 1 hour before the start of folding. In the other batches, which contained the cysteine in the form of the preservative, this was no longer necessary.

The reaction to give the prepro form of human insulin or prepro form of the insulin derivative was determined by means of HPLC.

HPLC analysis:

0.5 g of protein is dissolved in 40 ml of a solution of 6 M guanidine hydrochloride, 50 mM tris, pH 8.5 [lacuna] mM ethylenediaminetetraacetate (EDTA), 1% 2-mercaptoethanol and 10 mM dithiothreitol at 95°C for 2 min and then centrifuged at 14,000 g for 20 min. 0.02 ml of the clear supernatant is applied to a high-pressure liquid chromatography column.

Column: ®Nucleogel RP 300 – 5/46 (Macherey & Nagel, Aachen, Germany)
Gradient: buffer A: 0.1% trifluoroacetic acid (TFA)
buffer B: 0.09% TFA in acetonitrile
Temperature: 55°C
Total running time: 40 min.

The gradient is defined by the following amount of buffer B after the corresponding running times: 10 min 25%, 12 min 60%, 13 min 90%, 15 min 100%.

Flow: 1 ml/min

Detection: 215 nm

Result of the experiments

Table 1: Storage of fusion protein of human insulin at 5°C, valuable substance (mg/l) after folding (valuable substance means folded human insulin)

	1st day	1 week	2 weeks	4 weeks	8 weeks
With cysteine	850	831	867	845	825
Without cysteine	879	827	790	712	625

Table 2: Storage of fusion protein of human insulin at room temperature, valuable substance (mg/l) after folding

	1st day	1 week	2 weeks	4 weeks	8 weeks
With cysteine	792	817	800	785	790
Without cysteine	812	654	312	%*	%*

*Application not possible because of severe putrefaction processes

Table 3: Storage of fusion protein of the insulin derivative at 5°C, valuable substance (mg/l) after folding (valuable substance means folded insulin derivative)

	1st day	1 week	2 weeks	4 weeks	8 weeks
With cysteine	590	553	540	573	552
Without cysteine	612	580	549	518	404

Table 4: Storage of fusion protein of the insulin derivative at room temperature, valuable substance (mg/l) after folding

	1st day	1 week	2 weeks	4 weeks	8 weeks
With cysteine	643	667	680	649	653
Without cysteine	597	547	420	271	%*

*Application not possible because of severe putrefaction processes

By means of addition of cysteine or cysteine hydrochloride, storage times of up to 2 months are made possible without significant loss of activity.

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CLAIMS

1. A process for the storage of a protein in an aqueous solution, comprising adding cysteine to the aqueous solution in a concentration of from about 100 mM in order to delay the temporal decrease in the effective concentration of the protein during storage.

2. The process as claimed in claim 1, wherein the protein is a heterologous protein prepared in a microorganism.

3. The process as claimed in claim 2, wherein the microorganism is a bacterium.

4. The process as claimed in claim 3, wherein the bacterium is *Escherichia coli*.

5. The process as claimed in claim 2, wherein the microorganism is a yeast.

6. The process as claimed in claim 5, wherein the yeast is *Saccharomyces cerevisiae*.

7. The process as claimed in claim 5, wherein the yeast is *Pichia pastoris*.

8. The process as claimed in claim 2, wherein the protein is prepared from an expression vector construct.

9. The process as claimed in claim 1, wherein the protein is a heterologous protein and is prepared in an insect cell.

10. The process as claimed in claim 1, wherein the protein is present in dissolved form.

11. The process as claimed in claim 1, wherein the protein is present in suspension.

12. The process as claimed in claim 1, wherein the concentration of cysteine in the aqueous protein solution is in the range from about 100 mM to about 500 mM.

13. The process as claimed in claim 12, wherein the concentration of cysteine in the aqueous protein solution is in the range from about 150 mM to about 220 mM.

14. The process as claimed in claim 12, wherein the concentration of cysteine in the aqueous protein solution is about 170 mM.

15. The process as claimed in claim 12, wherein cysteine is added in a concentration of 500 mM to the aqueous solution.

16. The process as claimed in claim 1, wherein the storage of the protein takes place at about 0° C. to about 50° C.

17. The process as claimed in claim 16, wherein the storage of the protein takes place at about 5° C. to about 30° C.

18. The process as claimed in claim 17, wherein the storage of the protein takes place at about 5° C.

19. The process as claimed in claim 1, wherein the protein stored is insulin, an insulin derivative, or a precursor thereof.

20. A process for the preparation and storage of a heterologous protein, comprising the expression of the heterologous protein or its precursor in a transformed microorganism, optional disruption of the microorganism and/or isolation of the heterologous protein or its precursor from the culture medium, and the subsequent storage of the heterologous protein according to the process of claim 1.

21. The process of claim 20, further comprising the renaturation of the heterologous protein or its precursor and the purification and isolation of the heterologous protein, including optional removal of a leader sequence or other sequences that may be present in the precursor of the heterologous protein.

22. The process as claimed in claim 21, wherein the animal insulin is human insulin.

23. The process as claimed in claim 20, wherein the heterologous protein is animal insulin.

24. The process as claimed in claim 1, wherein cysteine is added in a concentration of greater than 100 mM to the aqueous solution.

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HMR 1999/L 042 WO

Abstract

The present invention relates to a process for the storage of proteins in an aqueous solvent. The addition of cysteine delays the decrease in the effective concentration of the protein. The process is suitable for use in the production of proteins in microorganisms.