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(54) PROTEINE DE FIBRE ET SA PRODUCTION
(54) FIBROUS PROTEINS AND THE PRODUCTION THEREOF

(57) L'invention concerne un procédé de production d'une protéine de fibre. Ce procédé consiste à: (a) exprimer une protéine de fibre précurseur d'une cellule végétale et (b) à incuber cette protéine de fibre précurseur avec une protéine traitant cette dernière. L'invention concerne également les cellules végétales utilisables à cet effet et les protéines de fibres obtenues par ce procédé.

(57) The present invention relates to a method for the production of a fibrous protein, comprising the following steps: (a) expression of a precursor fibrous protein in a plant cell and (b) incubation of the precursor fibrous protein with a protein processing the latter. The invention also relates to the plant cells used for this purpose and to the fibrous proteins produced according to the inventive method.

Abstract of the Disclosure

The present invention relates to a process for the production of a fibrous protein, comprising the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

Furthermore, this invention concerns plant cells usable for this purpose and fibrous proteins obtained by this process.

Fibrous Proteins and Their Production

The present invention relates to a process for the production of fibrous proteins in plant cells, plant cells usable for this purpose and fibrous proteins obtained by the process.

Fibrous proteins are proteins having mechanical stability, e.g. resilience or elasticity. They form from precursor fibrous proteins which are polymerized and cross-linked, respectively. This requires the presence of repetitive amino acid sequences in the precursor fibrous proteins and the influence of proteins which process precursor fibrous proteins. Fibrous proteins are found in animal and human cells. Examples of fibrous proteins are collagen and elastin. Both are components of connective tissues, e.g. skin, tendons, ligaments and blood vessels. Collagen forms by cross-linkage of tropocollagen molecules, while elastin is formed by cross-linkage of tropoelastin molecules.

Fibrous proteins are used for medical purposes and cosmetic purposes, respectively. To this end, they are frequently isolated from animal cells. This involves a great risk, since animal diseases, e.g. BSE, can be transmitted to man in this way.

Therefore, it is the object of the present invention to provide a process by which fibrous proteins can be produced without the above risks.

According to the invention this is achieved by the subject matters defined in the claims.

The present invention is based on the applicant's findings that precursor fibrous proteins can be produced in plant cells, which can then be converted into the corresponding fibrous proteins by treatment with proteins processing them. In particular, he found that precursor fibrous proteins can be produced in both individual plant cells and plants. He also discovered that the conversion of precursor fibrous proteins into the corresponding fibrous proteins can be made *in vitro* and *in vivo*. In the latter case, this can be made e.g. in that the precursor fibrous protein is expressed in a plant cell together with the protein processing it. The applicant made his discoveries using individual plant cells and plants, particularly the potato plant.

According to the invention the applicant's findings are used for a process for the production of a fibrous protein, which comprises the following steps:

(a) expression of a precursor fibrous protein in a plant cell, and

(b) incubation of the precursor fibrous protein with a protein processing it.

The expression "fibrous protein" comprises a fibrous protein of any kind and origin. It may have a two-dimensional or three-dimensional cross-linked structure. It can also be an animal or human fibrous protein. In addition, it may be available in wild-type or modified form. The latter comprises a fibrous protein whose amino acid sequence is modified as compared to the wild-type sequence at one or more sites. Such modifications may be additions, substitutions, deletions and/or inversions of one or more amino acids. In particular, amino acids may be present which are preferably expressed in plant cells. Besides, the fibrous protein may be a fusion protein, the fusion partner being e.g. oleosin. This protein then enables the localization of the fibrous protein in the oil phase of vegetable multiplication material. Fibrous proteins which are available in modified form have mechanical stability, e.g. resilience or elasticity, which is at least comparable to that of the wild-type form. Preferred fibrous proteins are collagen and elastin as well as derivatives and fragments thereof, respectively. As regards a modified form, the above statements apply to them correspondingly.

The term "expression of a precursor fibrous protein" comprises any expression of a gene coding for a precursor fibrous protein in a plant cell, the precursor fibrous protein being convertible into the corresponding fibrous protein as usual, e.g. by cross-linkage or polymerization. The above statements made on the expression "fibrous protein" apply here correspondingly. In addition, the precursor fibrous protein can be present with or without signal peptide. The former may be e.g. the natural or a foreign signal peptide, so that an extracellular localization of the precursor fibrous protein is enabled. In the latter, however, localization of the precursor fibrous protein is achieved in the cytoplasm. In addition, the precursor fibrous protein may have a control peptide so as to enable localization of the precursor fibrous protein in certain compartments of the plant cell, e.g. ER, chloroplasts or vacuoles. Preferred precursor fibrous proteins are tropocollagen and tropoelastin as well as derivatives and fragments thereof, respectively. For the expression of a gene coding for a precursor fibrous protein it is possible to use conventional expression vectors for plant cells. They comprise regulatory elements, e.g. enhancer, promoter and termination sequences detected in plant cells. Examples thereof are CaMV 35S promoter and termination sequences (cf. Odell, J.T. et al., Nature 313 (1985), 810-812). The expression vectors may also contain selection markers, e.g. a neomycin or kanamycin resistance gene. In addition, the expression vectors may contain sequences which favor their introduction into plant cells. For example, the expression vectors may contain T-DNA of binary vectors, such as pSR 8-30 or pSR 8-35/1, when they shall be introduced into plants *via Agrobacterium tumefaciens* (cf. Düring, K. et al., Plant Journal 3 (1993), 587-598; Porsch, P. et al., Plant Molecular Biology 37 (1998), 581-585). Besides, the expression vectors can also be introduced into plant cells by means of processes for which they do not require any special sequences. Such processes are e.g. microinjection, electroporation, DNA transfer by means of polyethylene glycol, liposome fusion or particle gun.

The expression "plant cell" comprises plant cells of any kind and origin. It may refer to individual plant cells, freshly isolated or established as a cell line, or those present in an aggregation. The latter is e.g. a plant or part thereof. Examples of plants are monocotyl plants, such as corn, rice, wheat, barley and sugarcane, and dicotyl plants, such as potato, tobacco, tomato, tea, coffee, brassicaceaen, particularly rape and cabbage, and leguminaes, particularly pea, phaseolus, vicia and soybean.

The expression "protein processing precursor fibrous protein" comprises any protein which can convert a precursor fibrous protein into the corresponding fibrous protein. The conversion can be made as usual, e.g. by cross-linkage or polymerization. Examples of such a protein are lysine oxidases. Also, proteinases may be concerned which, e.g. in the case of collagen, have been described. The lysine oxidases and proteinases, respectively, may be present as such and as derivatives or fragments thereof, respectively. The above statements made on a modified form of a fibrous protein apply correspondingly to them.

The expression "incubation of a precursor fibrous protein with a protein processing it" comprises any incubation of these proteins by which the precursor fibrous protein can be converted into the corresponding fibrous protein. The incubation may be made e.g. *in vitro*. For this purpose, it is favorable to incubate the expressed precursor fibrous protein in solution with the protein processing it. The incubation can also be carried out *in vivo*. For this purpose, it is favorable to express not only the precursor fibrous protein but also the protein processing it in a plant cell. Both proteins can be expressed in different plant cells which are then combined whereby the precursor fibrous protein is incubated with the protein processing it. The precursor fibrous protein and the protein processing it can also be expressed in the same plant cell. Thus, both proteins are automatically incubated in this plant cell. The above statements made on the expression of a precursor fibrous protein apply correspondingly to the expression of a protein processing a precursor fibrous protein.

A further subject matter of the present invention relates to a plant cell which expresses a precursor fibrous protein and a protein processing it. Also, a plant cell is preferred which expresses only the latter of these proteins. Regarding the expressions "plant cell", "precursor fibrous protein" and "protein processing precursor fibrous protein" reference is made to the above statements. In addition, the plant cell may be available in the form of a multiplication material.

Common methods can be used for the production of a plant cell according to the invention. In supplement to the above statements, the production of a plant according to the invention which expresses a precursor fibrous protein, e.g. tropoelastin, and a protein processing it, e.g. lysine oxidase, is described by way of example. In this connection, it is favorable to provide a cDNA coding for tropoelastin with CaMV 35S promoter and termination sequences and insert it in a binary vector, e.g. pSR 8-30 and pSR 8-35/1, respectively. The same can be done with a cDNA coding for a lysine oxidase. The resulting DNA molecules are used for transforming bacteria, e.g. E. coli S17-1 which are suitable for a transfer of the DNA molecules to *Agrobacterium tumefaciens*, e.g. GV 3101. For this purpose, E. coli S17-1 and *Agrobacterium tumefaciens* GV 3101 are mixed with each other and incubated overnight. Agrobacteria which have taken up the DNA molecules are selected by growth on carbenicillin-containing medium. They are then applied to cut-off potato plant leaves whose middle ribs were scratched several times and incubated in the dark for two days. Thereafter, the agrobacteria are removed and growth promoters are added to the potato plants, so that sprouts grow. They are cut off and used for cultivating new potato plants. The detection of the expression products tropoelastin and lysine oxidase and/or the resulting elastin is made by means of specific antibodies against these proteins. Reference is made to the below examples.

By means of the present invention it is possible to produce fibrous proteins in plant cells, particularly plants, in high purity. Therefore, the fibrous proteins are suitable for the most varying applications. They are found e.g. in agriculture, chemistry, production of cosmetics and medicine. In the latter case, e.g. the use of fibrous proteins for transplants and wound closures has to be mentioned. In particular, the fibrous proteins distinguish themselves in that they are free from animal or human viruses and pathogens, respectively. Moreover, the fibrous proteins can be produced in huge amounts. This applies particularly when they are isolated from plants cultivated in fields. Thus, the present invention represents a great contribution to providing pharmaceutical preparations safely and in great amounts.

The invention is explained by the below examples.

Example 1: Production of elastin in potato plants

A cDNA is used for human elastin (cf. Fazio, M.J., Journal of Investigative Dermatology 91 (1988), 458-464). This cDNA is provided with an NcoI restriction site at its 5' end and with an XbaI restriction site at its 3' end by means of PCR. The resulting cDNA fragment is inserted in the vector pRT 100 which contains an expression cassette having CaMV 35S promoter and termination sequences (cf. Töpfer, R. et al., Nucleic Acids Research 15 (1987), 5890; Odell, J.T. et al., above). Following cleavage using HindIII, the expression cassette containing the elastin cDNA is isolated and inserted in the binary vector pSR 8-30 (cf. Düring, K. et al.; Porsch, P. et al., above). The expression vector pSR 8-30 elastin is obtained.

In addition, a cDNA for human lysine oxidase is used (cf. Hämäläinen, E.R., Genomics 11 (1991), 508-516). It is treated as described above and inserted in the binary vector pSR 8-30. The expression vector pSR 8-30 lysine oxidase is obtained.

The expression vectors pSR 8-30 elastin and pSR 8-30 lysine oxidase are used for transforming E. coli S17-1. The transformants are mixed with *Agrobacterium tumefaciens* GV 3101 and incubated at 27°C overnight (cf. Koncz, C., Shell, J., Molecular and General Genetics 204 (1986), 383-396; Koncz, C. et al., Proc. Natl. Acad. Sci. U.S.A. 84 (1987), 131-135). Selection on carbenicillin is carried out, the bla gene necessary for this purpose being present in the above expression vectors. Selection clones of *Agrobacterium tumefaciens* are applied to cut-off leaves of potato plant cv. or named Désirée, whose middle ribs had been scratched several times and the plant is incubated in the dark at 20°C for 2 days. Thereafter, the agrobacteria are separated and growth promoters are added to the potato plant, so that sprouts form preferably. Moreover, non-transformed cells of the potato plant are killed by the addition of kanamycin to the plant medium. Rising sprouts are cut off and are allowed to grow roots on medium without plant growth substances but with kanamycin. The potato plants are further cultivated as usual.

The analysis of the expressed tropoelastin and lysine oxidase and/or the resulting elastin is achieved by antibodies in Western blot and ELISA, respectively, which are specific to the individual proteins. For this purpose, whole protein or the intercellular wash liquid of the potato plant is isolated and used in the corresponding detection methods.

It shows that tropoelastin and lysine oxidase can be expressed in plant cells, particularly in a plant. Moreover, it shows that by the incubation of lysine oxidase with the tropoelastin the latter is converted into elastin which can be isolated in pure form.

Example 2: Production of collagen in potato plants

cDNAs are used which code for the subunits α1 and α2 of human tropocollagen (cf. Chu, M.L. et al., Journal of Biological Chemistry 260 (1985), 2315-2320; Dickson L.A. et al., Nucleic

Acids Res. 13 (1985), 3427-3438). Furthermore, cDNAs are used which code for human lysine oxidase, human procollagen C proteinase and procollagen N proteinase, respectively, from bovine animals (cf. Hämäläinen, E.R. et al., above; Li, S.W. et al., Proc. Natl. Acad. Sci U.S.A. 93 (1996), 5127-5130; Colige, A. et al., Proc. Natl. Acad. Sci. U.S.A. 94 (1997), 2374-2379).

These DNAs are treated as described in Example 1 and inserted in the pSR 8-30 vector. The expression vectors pSR 8-30 tropocollagen $\alpha 1$, pSR 8-30 tropocollagen $\alpha 2$, pSR 8-30 lysine oxidase, pSR 8-30 C proteinase and pSR 8-30 N proteinase are obtained. The procedure is continued as described in Example 1.

It shows that tropocollagen and proteins processing it can be expressed in plant cells, particularly in a plant. In addition, it shows that collagen having a high degree of purity can be obtained.

Claims

1. A process for the production of a fibrous protein, comprising the following steps:
   (a) expression of a precursor fibrous protein in a plant cell, and
   (b) incubation of the precursor fibrous protein with a protein processing it.

2. The process according to claim 1, wherein the processing protein is expressed in a plant cell.

3. The process according to claim 2, wherein the precursor fibrous protein and the protein processing it are expressed in different plant cells.

4. The process according to claim 2, wherein the precursor fibrous protein and the protein processing it are expressed in the same plant cell.

5. The process according to any one of claims 1 to 4, wherein the plant cell is available in the form of a plant.

6. The process according to any one of claims 1 to 5, wherein the precursor fibrous protein is a procollagen or a derivative and fragment thereof, respectively.

7. The process according to any one of claims 1 to 5, wherein the precursor fibrous protein is a tropoelastin or a derivative and fragment thereof, respectively.

8. The process according to any one of claims 1 to 6, wherein the fibrous protein is a collagen or a derivative and fragment thereof, respectively.

9. The process according to any one of claims 1 to 5 and 7, wherein the fibrous protein is an elastin or a derivative and fragment thereof, respectively.

10. The process according to any one of claims 1 to 9, wherein the protein processing precursor fibrous protein is a lysine oxidase.

11. A plant cell, expressing a precursor fibrous protein and a protein processing it.

12. The plant cell according to claim 11, wherein the plant cell is available in the form of a multiplication material.

13. The plant cell according to claim 11, wherein the plant cell is present in the form of a plant.

14. The plant cell, expressing a protein processing precursor fibrous protein.

15. The plant cell according to claim 14, wherein the plant cell is available in the form of a multiplication material.

16. The plant cell according to claim 14, wherein the plant cell is available in the form of a plant.

17. Use of the plant cell according to any one of claims 11 to 16 for the production of a fibrous protein.

18. The fibrous protein, produced according to the process as defined in any one of claims 1 to 10.

19. The fibrous protein according to claim 18, wherein the fibrous protein is a collagen or a derivative and fragment thereof, respectively.

20. The fibrous protein according to claim 18, wherein the fibrous protein is an elastin or a derivative and fragment thereof, respectively.