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(54) Title: MODIFIED PROTEINS COMPRISING A BIOACTIVE PEPTIDE/PROTEIN LINKED TO A SEQUENCE OF AMINOACIDS CONTAINING A HUMAN CARBOHYDRATE BINDING MODULE (CBM), AND DEVELOPMENT OF A SYSTEM FOR THE ADMINISTRATION OF THERAPEUTICALLY ACTIVE PROTEINS AND RESPECTIVE UTILIZATIONS FOR BIOMEDICAL PURPOSES

(57) Abstract: It has been developed a strategy to simultaneously improve the properties of starch-based biomaterials for biomedical applications and the efficiency of therapeutical proteins. This new strategy which is revealed in this invention consists of the fusion of a carbohydrate binding module (CBM) with bioactive peptides. The human CBM used in this work has starch affinity (SBM). This SBM was identified as a module of a laforin enzyme, a phosphatase, which has the function to attribute glycogen affinity. As has been demonstrated the SBM does also have starch affinity. This is a low cost polysaccharide available in large quantities and with different properties which has increasingly raised interest for the development of biomedical applications. The biocompatibility, versatility and biodegradability are relevant starch characteristics for the development of applications as the controlled release of drugs and tissue engineering.

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Description

MODIFIED PROTEINS COMPRISING A BIOACTIVE PEPTIDE/ PROTEIN LINKED TO A SEQUENCE OF AMINOACIDS CONTAINING A HUMAN CARBOHYDRATE BINDING MODULE (CBM), AND DEVELOPMENT OF A SYSTEM FOR THE ADMINISTRATION OF THERAPEUTICALLY ACTIVE PROTEINS AND RESPECTIVE UTILIZATIONS FOR BIOMEDICAL PURPOSES

Field of invention

[1] This invention relates to chimerical and multifunctional proteins and the respective method of production. These proteins include a human carbohydrate binding domain (CBM), specifically with starch affinity (SBM). This invention also relates to the use of these chimerical proteins for biomedical applications, namely tissue engineering and controlled delivery of therapeutic proteins.

Background of the Invention

[2] The Carbohydrates Binding Modules (CBMs) are functionally independent modules, frequently found in nature associated to proteins involved in biomass breakdown. These modules are defined as sequences of aminoacids, present in enzymes which act on carbohydrates, exhibiting tri-dimensional structure and carbohydrates binding ability. The first CBMs to be found had cellulose affinity: the Cellulose Binding Domains (CBDs). Meanwhile, new modules, present in enzymes which act on carbohydrate substrates, other than cellulose, have been discovered. CBMs are also present in superior organisms. Initially, the CBMs classification was based on the aminoacids sequence homology. Today, the classification of glycosil-hydrolases is the ground for the CBMs families (currently 42), as classified and described by Coutinho and Henrrissat (http://afmb.cnrs-mrs.fr/CAZY/index.html). Depending on the CBM type and substrate characteristics, the adsorption may be either reversible or irreversible. The potential of CBMs in different fields of biotechnology has been demonstrated. One of these applications, relevant for the present work, relates to protein purification. The expression of proteins fused to CBDs, retaining their functional and specific properties has already been done successfully. As these studies demonstrated, CBDs may be used as probes with high cellulose affinity, allowing the isolation of bioactive peptides. The fusion of binding domains with, for instance, the stem cell factor (SCF) allowed the binding of this protein, by adsorption, to crystalline cellulose, retaining its potential for interaction with target cells (Doheny *et al.*, 1999).

The authors of this study concluded that the CBD-SCF linked to cellulose is actually more effective than soluble SCF, with regard to the presentation to target cells. In another study, it has been demonstrated that cells immobilization on a cellulose surface is favoured by the presence of recombinant *C.fimi* CBMs fused to RGD sequences. These results support the concept that CBMs have high potential as tools for the activation of biomaterials (Levy and Shoseyov, 2002).

[3] The biomaterials used for tissue engineering must have the capacity to induce tissue regeneration and its functionality. To date, the scaffolds available do not allow the fast and satisfactory regeneration of tissues. In spite of the current availability of growth factors and systems for controlled release of proteins, still there is the need for effective systems which favour cell migration and growth in tri-dimensional structures, through the use of adhesion and growth factors.

[4] Tissue engineering is a fast growing field of biomedical engineering. It requires the contribution from several fast growing areas of knowledge, namely cellular and molecular biology, materials science, chemical and mechanical engineering. The binding of cells to biomaterials, and its subsequent surface propagation, is mediated by glycoproteins present in the extracellular matrix (ECM). These proteins contain sequences that favour cell adhesion. These sequences interact with receptors in the surface of the cells, from the family of integrins. The ideal matrix for ECM substitution should include all the signals necessary for cells to grow, differentiate and interact, simultaneously constituting a mould for the production of the aimed structure, which degrades as the cells colonize the structure. The integration of bioactive molecules in this matrix is a central issue and one of highest interest (Hillery, 2001). The material present in the surface of the cells controls and influences several aspects of the cell physiology, such as adhesion, proliferation, activation, recruitment, migration and differentiation. The activation of a matrix surface (internal an external) used for cell culture, with highly adsorbed peptides to avoid its fast loss by diffusion, should allow a faster cellular adhesion and propagation. The matrix that supports the cellular growth may incorporate growth factors, allowing the controlled release of bioactive molecules. The tripeptide Arg-Gly-Asp (RGD) is an ubiquitous signal, present in several proteins responsible for cellular adhesion (Hersel *et al.*, 2003). The immobilization of different peptides with adhesive properties results in a selective cell response. CBMs provide a simple and effective way to direct bioactive peptides to a biomaterial surface, avoiding the use of complex peptide chemical grafting. According to Wetzel (1999), over a 100 recombinant therapeutic proteins are being developed. Examples of such bioactive proteins, which may be fused to CBMs, are the family of interleukins and morphogenetic bone proteins (BMPs), among others. Cytokines are molecular messengers which act locally, transmitting information between cells, with impact on the

regulation of growth, division, differentiation, inflammation and immunity. *In vivo*, cytokines are rapidly metabolized, by the liver and kidneys, after internalization through a receptor mediated process. This fast removal limits the scope and duration of its action. Interleukins promote its physiological action by binding to specific receptors on the cell surface, meaning that, exception made to cases where internalization is necessary, the immobilization of an interleukin in the surface of a biomaterial should not be an obstacle to its action. As a matter of fact, the fast removal of cytokines is recognized as one of the reasons for the limited success of the development of therapeutic applications of recombinant proteins (Asadullah *et al.*, 2003). The development of CBM-cytokine fusion proteins, spontaneously adsorbed to starch biomaterials, may be useful in stabilizing the bioactive proteins. The same applies to proteins such as BMPs, with high potential for bone regeneration/remodelling of bone tissue. These proteins are involved in the regulation of osteoblast growth and function, in the formation of bone tissue, with obvious application in the treatment of osteoporosis, osteoarthritis and fractures. The administration of soluble BMP not always gives rise to the expected bone regeneration, probably due to reduced BMP retention. Consequently, the development of new strategies for the progressive and controlled release of BMP, preferably over a large period of time, is necessary (Tanya, 1997).

[5] The controlled release of proteins may be achieved through the inclusion of the material to be used (nanoparticles, hydrogel, etc), using a CBM fused to the bioactive protein. Hydrogels are being tested for the controlled release of proteins, for instance, in assays of bone tissue growth stimulation (Yamamoto *et al.*, 2003), with encouraging results: the controlled release of BMP-2 from gelatine hydrogels, subcutaneously implanted, succeeded in inducing bone formation, as opposed to the much less effective administration of the protein through injection. In these assays, it has been shown that characteristics such as the gel porosity, water content, drug loading, all of these aspects influence the kinetics of protein release. By using CBMs, the system is simplified, since the protein is immobilized in the material surface, adsorbed through the CBM. In this system, the protein release is controlled through degradation/erosion, and only partially by diffusion, a mechanism which depends on the porosity and tortuosity of the support. This system may be very interesting, since frequently the materials used for tissue engineering have high porosity and interconnectivity. This property is essential for the viability of cellular growth within the scaffold, but inconvenient regarding the control of drug delivery, due to the high diffusivity of the drugs within the material.

[6] In the patent US 5 340 731 (1994), Kilburn and collaborators revealed the concept of using CBDs for protein purification. This strategy consists in the fusion of the protein of interest with a cellulose binding domain, linked through a peptide sequence

which includes a site for protease cleavage. The contact of the fused protein with an in-expensive stationary phase, cellulose, allows the fast immobilization and purification of the protein of interest, which is recovered using a protease. The same authors revealed a method which allows the *in vitro* expansion of cells that require growth factors for their expansion (patent US 5,874,308). This method consists on the utilization of growth factors fused to a CBM. This conjugate protein is immobilized through allowance to adsorb into a polysaccharide, through CBM, which is then added to the culture medium. The production of recombinant bioactive proteins, fused to a CBM, has been demonstrated in the work by Doheny and colleagues (1999). Specifically, the bioactivity of recombinant proteins such as CBD-SCF and CBD-RGD was demonstrated. In these studies, the Endoglucanase A (Cen A) CBD gene, from the bacteria *Cellulomonas fimi*, was used. The fusion protein CBD-RGD has a molecular weight of 17 kDa (Wierzba, *et al.*, 1995).

[7] The use of recombinant proteins CBD-RGD has been revealed in the US 6 407 208 patent (Chen, 2003), with the goal of substituting the highly expensive extra-cellular proteins used in the formulation of animal cells culture medium. The proteins from the extra-cellular matrix are used in the formulation of culture medium to improve the cellular adhesion. Most animal cells have the ability to adsorb to glycoproteins such as fibronectin, an interaction that favours the growth, adhesion and differentiation. Serum is a source of these glycoproteins (Barnes and Silnutzer, 1983), but it is difficult to isolate and expensive (Arathoon e Birch, 1986), which represents a limitation and a high cost in cultures of animal cells. The identification of alternative sources of the signals present in those proteins, would, thus, allow the reduction of the costs, and at the same time reduce the risks of microbial and viral infection. Thus, there is the need for the production of recombinant bioactive factors.

[8] With the actives peptides immobilized on the surface of the materials, the cells binding will be favoured. For example, the sequence RGD appears not only in the protein fibronectin but also in other glycoproteins from the extra-cellular matrix. If the sequence RGD, with the ability to promote cellular adhesion, is fused to a CBD and adsorbed on cellulose, the adhesion of cells to cellulose will occur. This way, the costs associated to animal cell culture are reduced, as well as the contamination risks. An invention revealed by Hsu (patent US 6 579 322), is based on the potential of the fusion proteins CBD-RGD to improve the biocompatibility of materials conceived for the production of biomedical devices. As a matter of fact, it has been demonstrated that the adhesion of endothelial cells to artificial blood vessels is promoted by the presence of the protein CBD-RGD coating the surface of the material. Shoseyov and colleagues revealed, in the patent US 5 496 934, a CBD from the bacteria *Clostridium cellulovorans* with high affinity for crystalline cellulose and chitin, as well as the methods

for its cloning and production. The methods for the production of CBD fused to a second protein has also been revealed and a vast range of applications of these CBDs and respective fusion products were claimed, including the controlled delivery of proteins, protein purification by affinity and diagnostic techniques.

[9] In another patent, (US 5 719 044), Shoseyov and colleagues claimed the use of CBD fusion proteins for applications such as the controlled delivery of proteins, protein purification by affinity and diagnostic techniques. This patent relates to the use of CBDs that bind to insoluble cellulose, with high affinity, with the goal of associating bioactive molecules to cellulose. Specifically, the goal of this invention is the production of a system for the controlled release of drugs, the drug being linked to a CBD, conserving the ability to adhere to cellulose. The linkage between CBDs and the drug may be carried out through chemicals such as a cyclohexylcarbodiimide, which may be used to establish amide or ester bonds.

[10] An European patent (EP1382681) describes also the potential of conjugates CBD-RGD to improve the efficacy of cell binding. The application envisaged in this work relates to the development of recombinant CBDs as serum substitutes, for animal cells culture, reducing the costs and contamination risks, simultaneously facilitating the purification of proteins expressed in the culture medium. All of the referred patents claim applications of non-human CBMs. On the contrary, this patent reveals the application of a human CBM, a relevant characteristic that may enlarge rather significantly the range of applications, namely through the fusion of the CBM with bioactive peptides. On the other hand, most of these patents relate to CBM with cellulose affinity, while in this case the polysaccharide that adsorbs the CBM is starch, a much more versatile material, considering biomedical applications.

[11] In another document (US 6,894,022), Hubbell and colleagues revealed the use of affinity probes for tissue repair and regeneration, and for the controlled drug release. The method introduced in this patent consists on the binding of heparin to a matrix (or hydrogel, used for the regeneration of tissues or for the controlled release of drugs); heparin is then linked, non-covalently, to growth factors. In the case of growth factors which do not spontaneously attach to heparin, a fusion protein including sequences with heparin-affinity may be created. The alternative use, as suggested in this work, of a human CBM with starch-affinity, offers considerable advantages over the use of heparin and respective affinity probes, because starch is a material available in large amounts, with low cost, available with different properties (molecular weight, ramification degree) and with low contamination risks. Besides, it may be included in the preparation and formulation of hydrogels, nanoparticles, etc.

Description of the invention

[12] According to the state of the art, the fusion proteins CBD-RGD have the potential

to promote cellular adhesion. The goal of the present invention relates to the use of recombinant adhesion factors SBM-RGD, or fusion proteins with the SBM fused to other bioactive peptides/proteins. Differently to the already available technologies, the present work introduces a human domain as the affinity tag. Being a human domain, with affinity for materials including starch in the formulation, new and relevant domains of application may be conceived for chimerical proteins comprehending a SBM, namely in the field of tissue engineering and the controlled release of protein. Being an human protein, although expressed in a bacterial host, the existence of an immunogenic action is unlikely, an aspect which represents a major advantage when compared to other CBMs (fungal or bacterial), proposed in other patents. On the other hand, starch being a material holding a great biomedical potential, considering its biocompatibility, reduced or null toxicity and presentation versatility. Furthermore, combined to other materials, starch may be obtained as an hydrogel, microparticles, nanoparticles, etc. Thus, the system now revealed offer a large range of potential applications in the biomedical area. The SBM may virtually be combined to any other biologically active protein, and administrated in a controlled way, for the development of a therapeutic action, using a convenient formulation that includes starch. Specifically, the applications considered in this work include the cultivation of human cells ex-vivo, preceding its implantation in the human body (tissue engineering). A gel containing adsorbed proteins SBM-RGD may be implanted, to allow the fast cell adsorption and proliferation.

[13] Another advantage related to this strategy is its simplicity. The adsorption of the protein onto the biomaterial occurs quickly, simply mixing the biomaterial in the presence of a solution containing the protein. The protein adsorption occurs quickly, and efficiently. The adsorbed protein may be retained in the biomaterial for relatively long periods, a process controlled by the material degradation kinetics. Thus, the biological activity in the biomaterial may be conserved for longer periods than in other systems where it is the diffusion of the material controlling the release kinetics.

Brief description of the figures

[14] Figure 1: Expression vector used to transform the *E. coli* strain BL21 (DE3).

[15] Figure 2: SDS-PAGE protein analysis: line 1- soluble fraction resulting from lysis of *E. coli* transformed with the vector pET25b-CBM-RGD; line 2- soluble fraction resulting from lysis of *E. coli* transformed with the vector pET25b; line 3- Precipitate resulting from lysis of *E. coli* transformed with the vector pET25b; line 4- Pellet resulting from lysis of *E. coli* transformed with the vector pET25b-CBM-RGD; MW- Molecular weight (KDa).

[16] Figure 3: SDS-PAGE protein purification: line 1- CBM-RGD purified using the system HisTrap™; MW - molecular weight (KDa).

Detailed description of the invention

[17] A method for the stimulation of tissue repair and regeneration, controlled release of drugs, particularly of therapeutic proteins, is described. The method makes use of starch-based scaffolds incorporating recombinant bioactive proteins exhibiting an SBM with starch affinity. Compared to other available systems, this method presents several advantages: the contact of the protein - fused to an SBM - with the starch matrix results in the highly efficient and fast adsorption of the protein; the protein immobilized by this technique has increased stability, and its release is controlled by the kinetics of the biomaterial erosion.

[18] The DNA sequence encoding the CBM was obtained by PCR, using the vector pET21a-laforin as a template. Other sequences, encoding bioactive peptides (RGD, BMP2), were fused to the CBM by PCR and/or cloning, using the convenient restriction enzymes. The CBM, CBM-RGD and CBM-BMP2 sequences were cloned in the expression vector pET 25b (+) (Novogen), which has an N-terminal *pelB* sequence, responsible for the periplasmatic secression, and an optional C-terminal HSV.Tag and His.Tag, which may be used for the detection and purification of the proteins. The vectors pET25b-CBM, pET25b-CBM-RGD and pET25b-CBM-BMP2 (figure 1) were used to transform the *Escherichia coli* strain BL21 (DE3).

[19] The recombinant strains were grown in Luria-Broth (LB) medium, supplemented with 50 µg/ml of ampicillin, at 37 ºC, with agitation, until the optical density, A_{600nm}, reached 0.6. The protein expression was induced with 1 mM of isopropyl-1-thio-β-D-galactopyranoside (IPTG), followed by the incubation for 16 h at 18 ºC. After the fermentation, the cells were recovered by centrifugation (15 min, 5000 rpm, 4ºC), and the precipitate was re-suspended in phosphate buffer (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.4), with 1 mM of phenylmetylsulfonyl fluoride (PMSF). The cell lysis was done by sonication. The lysate was centrifuged (30 min, 15000 rpm, 4 ºC). The recombinant protein (figure 2) was purified using the system HisTrap™ (figure 3) (Amersham bioscience).

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Claims

[1] Modified protein comprising a therapeutically active protein, linked to a sequence of aminoacids containing a human carbohydrate binding module (CBM), wherein the referred human module having starch affinity (SBM).

[2] Modified protein, according to the previous claim, wherein the therapeutically active protein being a cytokine, a growth factor, laminine, fibronectin, collagen or another biologically active protein.

[3] Modified protein, in according to the previous claims, wherein the bioactive peptide/protein containing the tri-peptide sequence Arg-Gly-Asp (RGD).

[4] Modified protein, according o the previous claims, wherein the starch binding module (SBM) being obtained from the human genome.

[5] Modified protein, according to the previous claim, wherein the module with starch affinity (SBM) being the N-terminal module, comprising 116 aminoacids, present in the human enzyme laforin.

[6] System for the controlled release of therapeutic proteins for biomedical purposes, according to the previous claims, characterized in that it consists on materials derived from starch having adsorbed proteins linked to the biomaterial through the SBM.

[7] System for the controlled release of proteins for biomedical purposes, according to the previous claim, wherein the biomaterial being a nanoparticle, a microparticle or an hydrogel, containing starch in the composition.

[8] System for the therapeutic administration of proteins for biomedical purposes, according to the claims 6 and 7, wherein the adsorbed peptide/ protein-SBM including the sequence RGD.

[9] Utilization of the modified proteins, according to the claims 1 to 5, characterized for being applied to the production of a system of therapeutic administration of proteins, described in the claims 6 to 8, consisting on pharmaceutical proteins linked to the biomaterial through the SBM adsorbed to starch or starch derived materials.

[10] Utilization of modified proteins, according to the previous claim, characterized for being destined to the production of a system for the therapeutic administration of proteins, as described in the claims 6 to 8, containing a human module linked to starch or starch derived materials.

[11] Utilization of a system, according to claims 6 to 8, characterized for being applied to the controlled administration of proteins for biomedical

purposes.

[12] Utilization of a system of modified proteins, according to the previous claims, characterized for being applied to the culture of cells *in vitro*, namely for tissue engineering.