Amino Acids (2004) 26: 419–423
DOI 10.1007/s00726-004-0086-4

Amino Acids
Printed in Austria

Producing transglutaminases by molecular farming in plants: Minireview article

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Received November 22, 2003
Accepted February 16, 2004
Published online June 17, 2004;

Summary. Transglutaminases have a range of catalytic activities, most of which concern the post-translational modification of proteins. The most important of these activities, both in terms of biology and biotechnology, is the cross-linking of proteins into large supramolecular networks. The widespread use of transglutaminases in research, medicine and industry has increased the demand for an inexpensive, efficient and safe source of recombinant enzymes. We describe initial results concerning the production of a mammalian transglutaminase in transgenic rice plants as a first step towards the large-scale molecular farming of this enzyme.

Keywords: Transglutaminase – Recombinant protein – Molecular farming – Plants – Biotechnology

Introduction

Transglutaminases are enzymes that catalyze the formation of covalent bonds through a reaction in which a glutamyl residue is the acyl donor and selected primary amines act as acyl acceptors (Griffin et al., 2002). These enzymes play an important role in protein modification and cross-linking, and are important for maintaining the structural integrity of tissues in both animals and plants. Due to their effects on the physical and chemical properties of proteins, they have many biotechnological applications particularly in the food processing industry, in medicine and in cosmetics (Collighan et al., 2002). Traditionally, the major industrial source of transglutaminase has been guinea-pig liver but for the last ten years this largely has been replaced by a microbial enzyme that can be produced by fermentation (Kuraishi et al., 1997). Transglutaminases from different sources have different activities and substrate specificities, so it would be valuable to have a production system that could be used to produce not only microbial enzymes but also those from animals and plants. The large-scale commercial production of enzymes and other technical proteins in plants has become feasible in the last few years and several efficient plant-based production systems have been developed (Twyman et al., 2003). In this brief review, we summarize the biological properties and industrial uses of transglutaminases, discuss the benefits of producing these enzymes by molecular farming in plants and describe our recent demonstration of the production of recombinant rat transglutaminase in transgenic rice.

Biological functions of transglutaminases

A large number of transglutaminases have been isolated from mammals and other animals, and the human genome contains genes encoding eight active transglutaminases as well as a catalytically inactive homolog that plays an important structural role in red blood cells (Lorand and Graham, 2003). Some of these enzymes are ubiquitous (e.g. TG2, TG7) while others are expressed in specific tissues (e.g. TG1, which is found predominantly in keratinocytes, and TG3, which is found in squamous epithelium and the brain). Within the cell, the enzymes are generally localized in the cytosol or at the plasma membrane, although many are also found in the extracellular

space where they are required for remodeling the extracellular matrix. Exceptionally, TG2 is found at several intracellular sites including the nuclear membrane, the nucleoplasm and mitochondria.

The major catalytic activity of the active enzymes is the Ca^{2+}-dependent formation of covalent bonds, resulting in either direct protein cross-linking via ε-(γ-glutamyl)lysine linkages thus forming isopeptide bonds or the conjugation of primary amines at glutamyl residues. The latter reaction can result in cross-linking if diamine or polyamines are used as molecular bridges, but they also form protein-polyamine conjugates as mono-γ-glutamyl-polyamine when only one primary amino group is involved in the conjugation (Griffin et al., 2002). Through a similar catalytic process, transglutaminases can carry out acylation, esterification, deamidation and isopeptide cleavage, although the biological relevance of these reactions is less clear. It is possible that such reactions are used to regulate protein structure, solubility or interactions with other molecules. For example, deamidation could be used to exchange a neutral glutamine side chain for a negatively charged glutamic acid, whereas amine incorporation could be used to add positively charged polyamine groups onto neutral glutamine residues (Lorand and Graham, 2003). Either of these changes would have a profound effect on the physicochemical properties of the protein and could be used as a regulatory mechanism. TG2 is a multifunctional enzyme that may be involved in signal transduction as well as protein modification.

Cross-linking reactions are important for a large number of biological processes in animals, from general functions such as assembling and remodeling the extracellular matrix to very specific processes such as formation of the skin barrier, hardening of the fertilization envelope and blood coagulation (the coagulation protein factor XIII is a transglutaminase). All these processes result in increased tissue strength and rigidity, and increased resistance to proteases. Competition between cross-linking and polyamidation reactions may help to control the way by which supramolecular protein assemblies are constructed and may thus regulate tissue construction, modeling and repair. Indeed, transglutaminases are essential in human development, playing important roles in the formation of the central and peripheral nervous systems, the heart, lungs and hematopoeitic system, and most importantly the skeleton and skin. The requirement for transglutaminases in cartilage modeling, ossification, skin development and maintenance is shown by the involvement of these enzymes in a number of severe skeletal and skin diseases, including lamellar icthyosis (Nemes et al., 2000). Transglutaminases are also involved in a variety of neurodegenerative, autoimmune and neoplastic disorders, the latter perhaps reflecting the fact that TG2 activity in humans is associated with apoptosis.

The exact relationship between transglutaminase activity and programmed cell death is unclear in part because the data are conflicting (De Laurenzi and Melino, 2001). Cross-linked protein networks are found in apoptotic bodies and transglutaminase activity has been shown to promote cell death in mice (Fesus et al., 1987; Melino et al., 1994; Aeschlimann and Thomazy, 2000), but other studies have shown that transglutaminase activity promotes cell survival (Grabarek et al., 2002). It is possible that transglutaminases have an accessory rather than regulatory activity, e.g. by preventing the inflammatory response to dead cells. In plants, increases in the levels of transglutaminase activity appear to correlate well with the onset of programmed cell death for example in tobacco flowers this can be delayed by spermine (Serafini-Fracassini et al., 2002), but it is notable that transglutaminases are also involved in normal cell division and, as is the case in animals, for cell differentiation.

Overall, much less is known about plant transglutaminases compared to their animal counterparts (Serafini-Fracassini et al., 1995; Serafini-Fracassini and Del Duca, 2002). Like the animal enzymes, plant transglutaminases are dependent on the presence of Ca^{2+} ions for their activity, but much of the research on the plant enzymes has focused on polyamine conjugation rather than protein cross-linking. In plants they are found at several different subcellular compartments, including the cytosol, cell wall, chloroplasts and mitochondria. Most data concern the chloroplast transglutaminase activity (Del Duca et al., 2000), which is regulated by light and known to modify Rubisco and several antenna proteins of photosystems I and II, influencing possibly the catalytic activity of the former and the energy transfer efficiency of the latter (Margosiak et al., 1990; Del Duca et al., 1994). More than one transglutaminase may be present in the chloroplast because at least two protein bands with different molecular weights have been identified in the chloroplast subfraction (Dondini et al., 2003). A transglutaminase activity has been detected in potato and mung bean mitochondria, and is enriched in the membrane fraction, but its role in this organelle remains to be established (Votyakova et al., 1999). Transglutaminases in the cytosol and plant cell wall are thought to play a structural role, particularly in terms of cytoskeletal rearrangement and the integrity of cell wall glycoproteins. In this respect it is notable that

transglutaminase activity is critical for the rapid growth of the pollen tube, which involves both cytoskeletal rearrangement and cell wall expansion (Del Duca et al., 1997), and during seedling growth, where it may be involved in the modification of seed storage proteins (Lilley et al., 1998). Transglutaminase activity is low during early G1 phase of the plant cell cycle but higher during late G1 and S phase, concomitant with increases in the levels of high molecular weight proteins. It is known that polyamines are required for cell cycle progression in both plants and animals, and this may be linked with the trend of transglutaminase activity (Del Duca et al., 2000).

Molecular farming of transglutaminases

At the current time, all transglutaminases are extracted from their native source, a process that is expensive, time-consuming and potentially hazardous when the enzyme is destined to be used in a therapeutic setting. Molecular farming provides an ideal solution because a safe expression host can be chosen and the protein can be produced in unlimited quantities under good manufacturing practice conditions. The industrial-scale production of foreign proteins can be achieved in many different expression hosts, including bacteria, yeast, cultured animal and plant cells, and transgenic animals and plants. Each of these systems has advantages and disadvantages, which must be evaluated on a case-by-case basis depending on the nature of the protein, its market value, the demand for the product and the time to market (Fischer et al., 2003). Recombinant protein expression in microbial cell cultures is advantageous due to the simplicity of gene transfer and the ease of scale-up. However, the requirement for expensive fermentation equipment and skilled technical personnel results in high set-up and maintenance costs. A more serious problem associated with bacterial cell cultures, particularly relevant to the production of human proteins, is the lack of post-translational protein modification in prokaryotic cells. For example, proteins produced in bacteria are not glycosylated (often leading to structural instability or loss of biological activity) and form disulphide bridges at a significantly slower rate than occurs in eukaryotes. The latter results in misfolding and precipitation of the protein in the form of inclusion bodies. Therefore, bacterial cultures are suitable for the production of simple and unmodified human proteins but not for large, complex glycoproteins. Some of the problems associated with protein modification can be overcome using yeast cultures, but there remain significant differences in glycosylation patterns between fungi and higher eukaryotes, making animal or plant expression systems necessary.

Traditionally, most recombinant human proteins have been produced in mammalian cell lines, such as CHO (Chinese Hamster Ovary) and NS0 murine myeloma cells (Chu and Robinson, 2001). These have the advantage of carrying out authentic post-translational modifications, but the costs of setting-up and running industrial-scale mammalian cell cultures can be prohibitive, particularly for industrial proteins required in bulk. As an alternative, transgenic animals expressing recombinant proteins in their milk or other body fluids have been developed, allowing proteins to be harvested periodically in a non-invasive manner. The yields are very high but gene transfer to domestic mammals such as sheep, goats, pigs and cows is not a trivial process, and scale-up is slow. There is generally a long development phase before protein production begins and there are many regulatory processes that have to be fulfilled. A disadvantage associated with all animal systems is the possible contamination of any recombinant protein product with an endogenous human pathogen, such as a virus or prion, or with oncogenic DNA sequences. The costs of protein farming in animal systems are therefore elevated by the expense of downstream processing to ensure the product is free from such contaminants.

Molecular farming in plants offers a unique combination of advantages in terms of cost, safety, efficacy and convenience (Twyman et al., 2003). The potential yield of recombinant proteins in transgenic plants is extremely high, and once the first generation of transformants has been produced and tested, scale up is rapid and inexpensive, simply involving the collection of seeds and the cultivation of more acres of land. This can be carried out with traditional farming practices, so there is no need for expensive equipment or skilled labor. Similarly, the collection and processing of transgenic crops involves traditional agricultural practices and existing infrastructure. Unlike transgenic animal systems, there is no need to maintain a founder herd carrying the transgene, since plant material can be stored as seed and then sown as required. Plants do not contain human pathogens, a fact that is proven by the consumption of plant material as food. Recombinant proteins can be targeted to storage organs such as seeds, where they remain stable for years even at ambient temperatures, reducing the costs of distribution and storage (Stöger et al., 2002). For industrial proteins, plant material can be added directly to the industrial process, eliminating the costs of extraction and

purification. This could be useful for example in food processing, brewing or in the paper industry.

Production of rat transglutaminase in rice

Many high value recombinant proteins with diagnostic, prophylactic or therapeutic potential have now been expressed in plants, and have been shown to retain their biological activities. While tobacco has been favored as an expression host due to its long history as a model organism and large harvest biomass, other species such as rice are also being evaluated. Rice has a number of advantages over tobacco reflecting the fact that recombinant proteins can accumulate in a stable form in the desiccated seed, allowing long-term storage and distribution at ambient temperatures (Stöger et al., 2002). We have therefore explored the possibility of using rice as an expression platform for a mammalian transglutaminase, the first time to our knowledge that this has been attempted in plants. We made a gene construct comprising the rat *DP1* gene (Ho et al., 1992) flanked by the maize *ubi*-1 promoter/first intron and the *nos* polyadenylaton signal. This construct was introduced into mature rice embryos by particle bombardment (Claparols et al., 2004). Transgene expression at the mRNA level was confirmed in leaf tissue by RT-PCR, while western blots demonstrated protein presence in the leaves. The addition of 5 mM $CaCl_2$ resulted in an up to five-fold increase in total transglutaminase activity in the transgenic plants, but no change was observed in non-transgenic controls. Our results show that the plant-derived rat transglutaminase is Ca^{2+} dependent and functional in an *in vitro* assay. We therefore established that rice plants could be used to produce a functional mammalian transglutaminase.

Conclusions

Transglutaminases are enzymes that catalyze cross-linking reactions between proteins. They are widely used in the food processing industry and in the fields of medicine and cosmetics. Current commercial sources of transglutaminase are expensive because the protein is either isolated from its natural source or produced by bacterial fermentation. Based on our experience in molecular farming, the production of valuable and therapeutically relevant proteins in plants, we have described for the first time transgenic rice plants containing and expressing the gene for a mammalian transglutaminase. Although the plants produced large amounts of transglutaminase, there was no change in phenotype suggesting that the enzyme accumulated in an inactive form. This is in agreement with the properties of prostatic gland transglutaminase that is normally inactive inside the cell. It becomes active upon secretion. *In vitro* assays showed that the recombinant enzyme, like its native counterpart, is dependent on the presence of Ca^{2+} ions for catalytic activity.

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