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Protease activity in the medium of larch (*Larix* spec.) embryogenic suspension cultures and medium-protein stabilization by compatible solutes

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Summary

The stability of recombinant, secreted protein in the medium of transgenic plant cell cultures heavily determines the resulting protein yield, which is a crucial factor for every production system. In order to gain more knowledge about the feasability of rapidly growing larch (*Larix* sp.) embrogenic cell cultures as a possible expression system for recombinant proteins, spent cell culture medium was characterized in this study. An accumulation of endogenous proteins could be observed in the medium of larch embrogenic suspension cultures which reached up to 1750 µg per g fresh weight. In contrast, low protease activity accumulated within a typical 14-day culture period in the medium. This activity was up to 20 times lower than the protease activity in two callus-derived suspension cultures of tobacco (genotype SR1 and BY-2) which were measured in parallel. To asses the stability of foreign proteins, medium aliquots were spiked with Immunoglobulin G (IgG) and the amount of protein degradation was determined after 23 h of incubation by SDS-PAGE. The loss of IgG was comparable in three different larch genotypes, resulting in a mean loss of 18 % during the incubation time. This loss could remarkably be diminished by the addition of ectoin derivatives, known to be protein-protective „compatible solutes“ of bacterial origin. The most effective one was hydroxyectoin which resulted in a 76 % reduction of the observed IgG degradation. The stabilization of proteins in plant cell culture medium by compatible solutes is shown here for the first time. The possible mechanism of the stabilizing effect is discussed.

Abbreviations:
2.4-D, 2.4-dichlorophenoxy acetic acid, CIM, callus induction medium, MS, Murashige and Skoog, PBS, phosphate buffered saline

Introduction

Plant cell suspension cultures may offer a viable alternative to established bacterial and animal cell culture systems for the production of recombinant therapeutic proteins since they are able to generate complex oligomeric proteins in a biologically active manner without the risc of human pathogenic contamination (Doran, 2000). Moreover, they can be grown on simple, cost-effective media under controlled conditions without the release of transgenic plants into the environment. The secretion of the expressed proteins into the medium is an important feature for it enables a simplified purification strategy (Fischer et al., 1999a) without the need to harvest and extract the cell material. This also is the basis for sophisticated bioreactor cultures using perfusion-, semi-continuous or continuous fermentation strategies (McDonald et al., 2005; Lee et al., 2004).
Plant cell culture media generally consist of inorganic compounds (nutrients and trace elements) and some organics like vitamins or sugar. This aqueous, low salt-environment can be, however, a critical factor for the stability of secreted proteins, resulting in inactivation or degradation after secretion (Tsoi and Doran; 2002, Sharp and Doran, 2001). Additionally, co-secreted proteases were detected in tobacco suspension cultures, causing loss of recombinant target protein (Schiermeyer et al., 2005; Kwon et al., 2003).
The recovery of protein stability is therefor one crucial parameter that heavily determines the overall yield of recombinant protein from plant *in vitro*-cultures. For this, several protein-protective agents have been added like gelatin (Kwon et al., 2003), polyvinylpyrrolidone (LaCount et al., 1997) or protease inhibitors (Bateman et al., 1996). Baur et al. (2005) have co-expressed human serum albumin to stabilize secreted recombinant protein. A new class of protein-protective substances are the compatible solutes (Kempf and Bremer, 1998; Galinski, 1993). These low-molecular-weight molecules are taken up or produced by halophilic bacteria to protect themselves against water stress in extreme environments. The protein-protective properties have been applied to plant cells by transformation with the relevant biosynthesis enzymes (Bohnert and Shen, 1999), but they never have been tested as a medium additive for plant cell cultures yet.
Embryogenic cultures of larch (*Larix* spec.) originated by direct somatic embryogenesis from zygotic embryos (Klimaszewska, 1989) are propagating rapidely in suspension culture (Jain, 1998; Silveira et al., 2004). Moreover, the genetic transformation could be established (Ismail et al., 2004; Taryono, 2000). This makes this type of plant material a promising tool for biotechnological application like mass propagation (Silveira et al., 2004; Archambault et al., 1994) or production of foreign proteins under controlled conditions (Doran, 2000).
To further test the suitablility of these cultures for protein production, the possible secretion of proteases into the medium over the culture period was tested in comparison with tobacco suspension cultures. Moreover, the stability of exogeneously added immunoglobuline G (IgG) was tested in spent culture medium aliquots. Finally, the influence of the addition of compatible solutes (ectoin, hydroxyection, homoectoin) to the medium on protein (IgG) stability was tested here for the first time.

Material and methods

Chemicals

Azocasein, imunnoglobulin G (technical grade, I 8640) and polyvinylpyrrolidone were purchased from Sigma (St. Louis, USA). Ectoin and homoectoin were from Bitop GmbH (Witten, Germany). Hydroxyectoine was synthesized at the Institute of Arteriosclerosis Research, university of Münster, Germany (Schnoor et al., 2004). All other chemicals were of the highest purity available.

Plant cell culture

Embryogenic cultures of *Larix decidua* Mill., genotypes 4/93 and S90, and *Larix x eurolepis* genotype 58 were maintained on MSG medium (Becwar et al., 1990) which is a modified Murashige and Skoog´s (MS) medium with vitamins but with decreased nitrate and sup-

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plemented with 9 µM 2.4-dichlorophenoxyacetic acid (2.4-D), 2.25 µM 6-benzylaminopurine (BAP), 10 mM L-glutamine and solidified with 0.3 % (w/v) Gelrite.
Suspension cultures were initiated by adding embryonal masses (3 g fw) to 50 mL liquid MSG-medium in a 300 mL Erlenmeyer-flask and maintaining on a rotary shaker at 80 rpm and 21°C ± 2°C in the dark. They were subcultured every 14 days.
Additionally, callus-derived suspension cultures of tobacco (*Nicotiana tabacum* L.) cv Petite Havana „SR1" and cv. Bright Yellow 2 (BY-2) have been used for control experiments. SR1 cells were cultivated in liquid callus induction medium (CIM), which consists of basal MS-medium (4.6 g/L MS-salts, 30 g/L sucrose, pH 6.3) supplemented with 0.5 mg/L 2.4-D. The genotype BY-2 was cultivated in liquid BY-2 medium, which consists of a modified MS medium (4.3 g/L MS-salts, 30 g/L sucrose, pH 5.8) supplemented with 0.6 mg/L thiamine/HCl, 200 mg/L KH_2PO_4 and 0.5 mg/L 2.4-D. Cultures were cultivated on a rotary shaker under identical conditions as decribed above.
For fresh weight determination, suspensions were passed over a sterilized nylon sieve (50 µm mesh width) to separate the cells from the medium. The plant cells were weighted after removel of residual liquid using sterilized filter paper

Protein preparation from medium

Medium of suspension cultures of *L. decidua* genotypes 4/93 and S90 was harvested 3, 7, 10 and 14 days after inoculation and the fresh weight was determined as decribed above. Protein quantification was performed by Bradford method (Bradford, 1976). 1 ml-aliquots of the media were concentrated by filtering over regenerated cellulose (Amicon Ultracel YM-10 columns, Millipore, Billerica, USA). The concentrats were adjusted to 21 µl with water and used for SDS-PAGE.

Soluble cellular protein extraction

For soluble cellular protein control, 0.8-1 g fresh weight of cell material of each genotype was ground in liquid nitrogen to a fine powder with mortar and pestle. Total soluble protein was extracted using 2 mL extraction buffer (100 mM 2-(N-morpholino) ethane sulfonic acid, pH 6.0, 1 mM dithiotheritol, 10 % (w/v) glycerol,) per g of tissue. Cell debris was removed by centrifugation at 14,000 rpm and 4°C for 15 min and the supernatant was the crude extract of soluble proteins. Protein quantification was done by Bradford method (Bradford, 1976).

Protease activity assay

Protease activity in the medium was determined by azocasein degradation after Sharp and Doran (2001). Briefly, 650 µL medium aliquots were incubated with 350 µL azocasein solution (1 % (w/v) azocasein in PBS) for 16 h at 25°C. The azocasein was precepitated by adding 500 µL of icecold 20 % (w/v) trichloro acetic acid. After centrifugation at 14,000 rpm at 4°C for 10 min, the supernatant was used for subsequent photometrical analysis at 340 nm. For background correction, a blank assay using fresh instead of spent medium was used and the value was substracted from spent medium measurements.

IgG stability assay

Medium aliquots of 500 µL each were taken from 7 days-old suspension cultures of all three genotypes. In experiments where somatic embryo material was included in the aliquots, a total of 650 µL was taken. After spiking each aliquot with 5 µg IgG, they were incubated for 6 or 23 h at 21°C on a rotary shaker at 80 rpm. For protein precipitation, two volumes of icecold ethanol were added and the samples were stored at -20°C overnight. After harvesting the protein by centrifugation at 14,000 rpm and 4°C for 30 min, the resulting pellet was washed with 70 % (v/v) ethanol, air-dried and resolved in 21 µL of water for subsequent analysis using SDS-PAGE.
For testing the influence of different additives on the IgG stability, medium aliquots were processed as described above, but samples were adjusted to 0.1 M ectoine, 0.1 M hydroxyectoine, 0.1 M homoectoine or 0.75 g/L polyvinylpyrrolidone before spiking the samples with 5 µg IgG.

SDS-PAGE and protein quantitation

6 µL of 5 x Laemli buffer (62.5 mM Tris/HCl pH 6.6, 2 % (w/v) SDS, 10 % (v/v) glycerol, 0.8 % (w/v) bromphenol blue) and 3 µL 0.5 M dithiothreitol were added to each sample which was denatured afterwards at 100°C for 5 min. Proteins were spearated on a 12.5 % SDS-PAGE with 25 mA per gel. After staining of the gels using coomassie brilliant blue (for spiking experiments) or silver staining protocol (for naturally secreted proteins), they were photographed using a HV C20A video camera (Hitachi, Japan).
For quantitation, band intensity was analyzed using Quantity One™ software version 4.2.1.(Biorad, Hercules, USA).
After background substraction, the intensity of the IgG heavy chain-bands of each sample was determined. The intensity of a prominent protein band of a medium protein of ca. 50 kDa that occurred in all media aliquots was used as an internal standard. Two IgG samples (2,5 and 5 µg) were also included in each gel and the resulting intensity of the heavy chain-bands was used to quantify the amount of IgG in the medium samples.

Results

Protein accumulation in the medium of embryogenic larch cultures

To preliminary clarify whether embryogenic cultures of larch at all secrete any natural proteins into the medium, samples of 1 mL were taken from genotypes 4/93, S90 and 58 3, 7, 10 and 14 days after inoculation and the protein content was determined. All three genotypes showed a time-dependend accumulation of proteins. The amounts, however, were highly genotype-dependent. At the end of the 14 day-culture, medium proteins had amounted to 110 µg/g fw (genotype 4/93), 320 µg/g fw (genotype S90) and 507 µg/g fw (genotype 58) (See Fig. 1 A). The protein content remained quite low during the first 10 days of culture in genotypes 4/93 and S90, indicating only slight protein secretion. The protein accumulation in the medium of the tobacco genotypes were comparable, resulting in 487 µg/g fw and 250 µg/g fw for SR1 and BY-2, respectively. On day 14, another 1 mL aliquot was concentrated and the total medium protein was subjected to SDS-PAGE. Fig. 1 B illustrates an example gel picture of larch genotypes 4/93 and S90 and the tobacco genotypes. Especially genotype 4/93 shows a wast variety of secreted proteins and its size partially exceeds 100 kDa. When compared with the protein pattern of equivalent amounts of soluble cellular proteins of cultured embryos, some clear differences could be detected. However, some of the bands indeed seem to have an equivalent in the soluble cellular protein fraction.

Protease activity in spent medium

In a first set of experiments, protease activity in the medium of the cultures was monitored over a standard culture time period of 14 days (Fig. 2). For this, a protease activity assay based on the digestion of azocasein was applied (Sharp and Doran, 2001). Medium aliquots were incubated with azocasein solution. After precipitaion, the

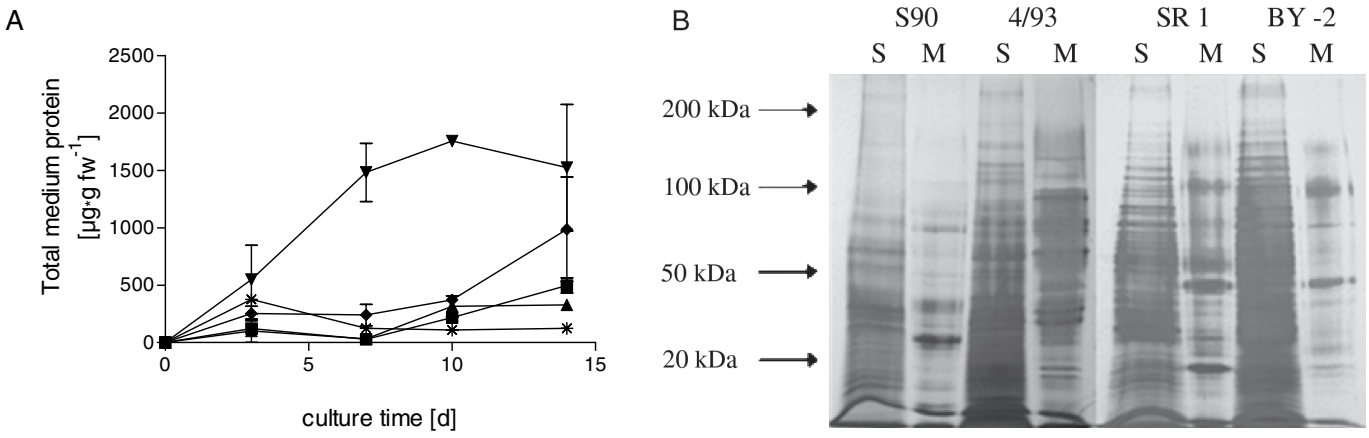

Fig. 1: Naturally secreted proteins by larch embryogenic culture. (A) Accumulation of total protein in the medium of *L. decidua* genotype 4/93(▲) and S90 (■), *L. x eurolepis* 58 (▼), *N. tabacum* genotype SR1 (♦) and BY-2 (✳) over a culture period of 14 days. The data are mean of 2 independent replicates. Error bars represent maximal divergence of values. (B) Representative silver-stained SDS-PAGE of 1 ml-medium aliquots of culture medium of genotypes S90, 4/93, SR1 and BY-2 (M) and the same protein amount of total soluble cellular proteins (S) 14 days after inoculation.

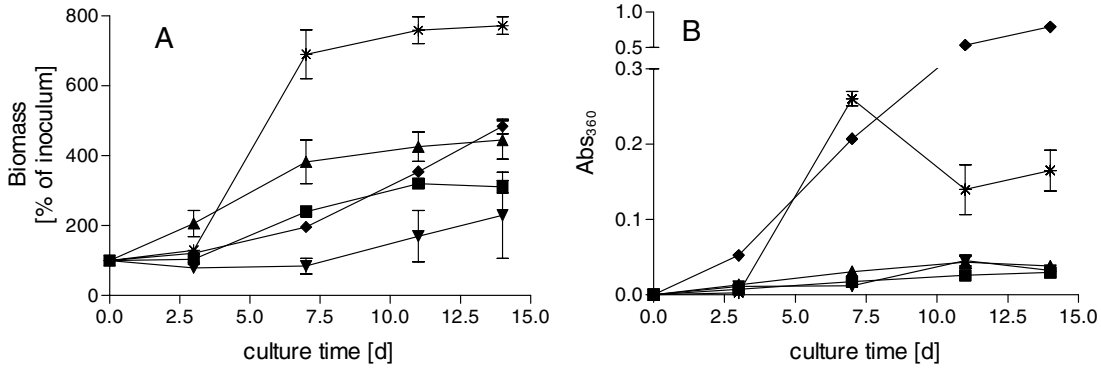

Fig. 2: (A) Growth rate and (B) protease activity in the medium of *L. decidua* genotype 4/93(▲) and S90 (■), *L. x eurolepis* 58 (▼), *N. tabacum* genotype SR1 (♦) and BY-2 (✳) over a culture period of 14 days. Protease activity was determined by azocasein assay as described in Materials and methods. The data are mean of 2-3 independent replicates. Error bars represent standard error.

supernatant contained the non-precipitable peptide fragments resulting from protease activity which can be quantified photometrically. The media of all three larch genotypes showed only minute amounts of protease activity which were slightly increasing over the culture time (Fig. 2 B). For comparison, the protease activity was also determined in tobacco suspension cultures of the genotypes SR1 and BY-2. Here, protease acitivity was reasonably higher. The genotype SR1 by far showed highest protease activity accumulation over time (Fig. 2 B). Here, protease activity was approx. 20 times higher at day 14 than the larch genotype 4/93, although the biomass was comparable. The medium of genotype BY-2 also showed a fast increase of protease activity during the exponential growth phase but then is decreasing during the stationary phase. When compared with the larch culture medium, BY-2 medium exhibits an approx. 6 times higher protease activity at the beginning of the stationary phase (day 7) compared to the mean of the genotypes 4/93 and S90 at the comparable phase (day11). Normalized to the same amount of biomass, the BY-2 medium still shows approx. 3 times higher protease activity per g fresh weight (data not shown).

Stability of IgG in spent culture medium

To evaluate the stability of secreted recombinant proteins in the medium of transgenic cell lines in future applications, immunoglobulin G (IgG) was added to spent medium aliquots from the exponential growth phase of the different cultures (7 days after inoculation). Spiked aliquots were incubated for 23 h and the protein was recovered afterwards by ethanol precipitation. A precipitation using methanol, desalting of the samples using sephadex-G-25 prior to precipitation or freeze-drying the samples instead of precipitation did not result in higher protein recovery from the samples (data not shown). To quantify and compare the recovered IgG, samples were subjected to SDS-PAGE. The resulting heavy chain-bands of the IgG were quantified according to their intensity in the coomassie-stained gels and compared to spiked samples without incubation time. Results are shown in Fig. 3. They clearly show a loss of exogeneously added IgG over the incubation time which was approx. 16 % (genotype S90), 18 % (genotype 58) and 20 % (genotype 4/93), respectively. The controls (no incubation time) differ from the originally added 5 µg IgG due to a non-quantitative ethanol-precipitation, which, however, is comparable in all controls. The control experiment using fresh instead of spent medium resulted in approx. 95 % recovery of the added IgG (data not shown), indicating that the effect measured in the spent medium cannot be due to unspecific sticking of the proteins to the tube walls during the experiment.

Effect of protein-protective medium additives

In order to find conditions of enhanced protein stability in spent medium of larch embryogenic cultures, polyvinylpyrollidone, ectoin

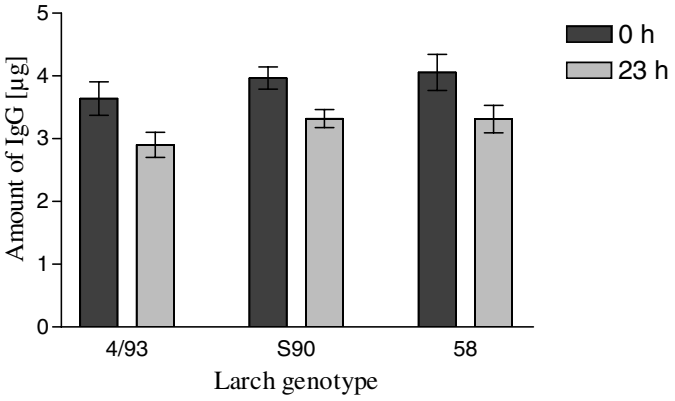

Fig. 3: Loss of exogenuously added immunoglobulin G (IgG) in spent larch embryo culture medium over 23 h. 7 day-old medium aliquots (0.5 ml each) were spiked with 5 µg IgG and incubated for 23 h. The proteins were ethanol-precipitated and the heavy chain was used for densitometric quantitation on SDS-PAGE. The data are mean of 5-11 independent replicates. Error bars represent standard error.

and two ectoin derivatives, hydroxyectoine and homoectoine, have been tested as medium additives. Basically, the same protein stability assay using IgG-spiked medium aliquots were deployed but with the addition of the mentioned substances. The results are summarized in Fig. 4, demonstrating that the stability of IgG could significantly be enhanced in media aliquots of all larch genotypes to a similar extent. Whereas the loss of IgG was 16-30 % without additives, the addition of 0.1 M ectoin could reduce this loss to a mean of 10 %. Similarly, the IgG loss was reduced to 9 % in the case of 0.1 M homoectoin. The addition of 0.1 M hydroxyectoin led to the best results, reducing the loss of IgG to a mean of 6 % per 23 h of incubation. This is in the range of the control using fresh medium (see above) and clearly demonstrates the usefullness of the addition of 100 mM hydroxyectoin to the culture medium of embryogenic larch cultures. The addition of 0.75 g/L PVP did not draw a homogeneous picture and resulted in an average reduction of IgG loss of only 15 %

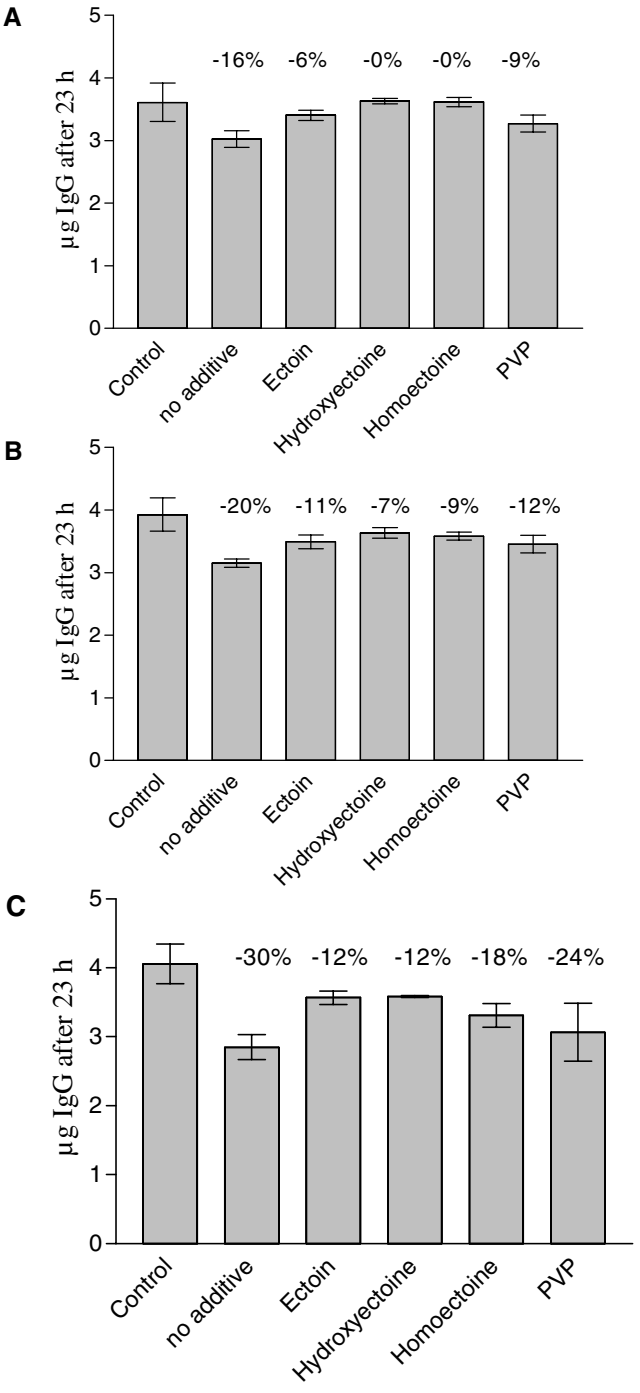

Fig. 4: Influence of different protein-protective additives to the stability of IgG in spent medium of larch embryogenic cultures of the genotypes (A) *L. decidua* 4/94, (B) *L. decidua* S90 and (C) *L. x eurolepis* 58. 5 µg IgG were exogeneously added to medium aliquots without additives or in the the presence of different ectoins (100 mM each final concentration) or 0.075 % (w/v) polyvinylpyrrolidone (PVP) and recovered after 23 h of incubation. Control : IgG addition and recovery without incubation time. The data are mean of 5-7 independent replicates. Error bars represent standard error.

Discussion

The overall protein yield is of paramount importance for an economic feasibility of cell culture-based expression systems. This yield is not only a function of gene expression level, but also of mRNA stability, efficiency of translation and post-translational modifications. The stability of the protein once secreted into the medium also greatly attributes to the protein yield. However, in the case of plant cell cultures, secreted proteins has been shown to be subject to degradation by co-secreted proteases (SCHIERMEYER et al., 2005; KWON et al., 2003), adsorption, aggregation or inactivation by interaction with anorganic media components (SHARP and DORAN, 2001) or adsorption to glass walls (TSOI and DORAN, 2002).

We therefore attempted to characterize the protein stability in the medium of larch embryogenic cultures, which may are an interesting production platform for recombinant proteins. This was done by means of protease activity determination and degradation assay of spiked IgG in spent cell culture medium. IgG was chosen as an example for a future target protein for genetically modified larch embryogenic strains.

In first preleminary experiments, we tested the medium for the occurrence of naturally secreted proteins and compared this pattern with the soluble cellular protein pattern. Whereas several bands have a size equivalent in the soluble cellular protein extract, some appear exclusively in the medium, thus are putative secreted proteins. The occurrence of natural protein secretion is widely recognized in callus-based suspension cultures, e.g. in sunflower (MITA et al., 1997) or lupin (WOJTASZEK et al., 1998; WINK, 1994), as well as in somatic embryo-based suspension cultures, e.g. carrot (VAN HENGEL et al., 2001). It is regarded as a common phenomenon for plant cell cultures

besides the secretion of low-molecular weight compounds like amino acids and organic acids (WINK, 1994). The secreted proteins have function in cell wall biosynthesis, cell signalling (VAN HENGEL et al., 2001; LOOPSTRA, 2004), pathogen defence or are stress-related (MITA et al., 1997; WOJTASZEK et al., 1998).

To further characterize secreted proteins in embryogenic larch cell cultures, a protease activity assay was performed which showed significant, but low amounts of protease activity in the medium of all three genotypes which slightly increases over the culture period. However, it remains questionable whether the detected protease activity results from the release of cytosolic or vacuolar proteases from destroyed cells or whether they are actively secreted. Although the medium was taken from cell in the exponential growth phase, there is always a little amount of dead cells within the population, as revealed by fluorescein-staining (unpublished observations). Comparative analysis of protease activity in tobacco suspension cultures (genotypes SR1 and BY-2) revealed a considerable higher amount and pronounced accumulation of protease activity which more probably results from secretion. The existence of proteases in these genotypes have also been observed by KWON et al. (2003) for SR1 and by SCHIERMEYER et al. (2005) for BY-2. In the latter, the oberserved decrease of protease activity in the stationary phase probably is due to protein degradation as was also noticed in lupin cell cultures (WINK, 1985).

In order to assess the stability of foreign protein in spent larch cell culture medium, we examined the fate of exogenuously added IgG in 7 days-old medium. After 23 h of incubation, the loss of IgG was at an average of 18 %. A decrease in secreted recombinant protein has also been reported in tobacco (SHARP and DORAN, 2001; BODEUTSCH et al., 2001) and tomato suspension culture (KWON et al., 2003b). This loss can be attributed to degradation by proteases as observed in tobacco cell cultures (KWON et al., 2003a; SHARP and DORAN, 2001b). A loss of protein can also be due to media component interaction (TSOI and DORAN, 2002). No degradation or aggregation products, however, could be observed. The loss of the added IgG cannot be attributed clearly to one of these possibilities since we were not able to recover the test protein from fresh-medium control samples.

When plant cell cultures are to be used as production systems for recombinant proteins, secretion of these proteins into the medium is of great importance to facilitate the dowenstream processing (FISCHER et al., 1999). Thus, several medium additives have been tried for enhancement of protein stability. One strategy is to add protease inhibitors (BATEMAN et al., 1997), but more common is the addition of substances which unspecifically stabilize the recombinant protein, using dimethyl sulfoxide (WAHL et al., 1995), gelatin or poylvinyl-pyrrolidone (WONGSAMUTH and DORAN, 1996; LACOUNT et al., 1997) or bovine serum albumin and salt (JAMES et al., 2000). Recently, protein stabilization was achieved by co-expression of human serum albumin in the moss *Physcomitrella patens* (BAUR et al., 2005).

To find out conditions where recombinant proteins secreted into the larch embryo medium are stabilized, we applied the protein stability assay to spent medium samples with addition of protein-protective substances. Besides PVP, a polymer known to stabilize proteins in aqueous solutions (LACOUNT et al., 1997), we tested members of the compatible solutes, a new class of protein-protective substances (GALINSKI, 1993; KEMPF and BREMER, 1998). Ectoins belong to compatible solutes of bacterial origin, which recently has been identified to occur in extremophilic bacteria like *Halmononas elongata* (SAUER and GALINSKI, 1998). Ectoin has been shown to support periplasmic protein expression in bacteria (BARTH et al., 2000) and to confer hyperosmotic tolerance in transgenic tobacco BY-2 cells that produce this compatible solute (NAKAYAMA et al., 2000). Ectoines never have been tested as medium additive in plant cell cultures, although the general protein-protective properties have been shown (GALINSKI et al., 2000). Here, we firstly report that ectoins successfully can recover protein stability when added to the medium in concentrations of 0.1 M. The addition of hydroxyectoin was most successful, resulting in 73 % reduction of the IgG loss over 23 h. These results indicate the usefulness of ectoin and ectoine derivatives as protein-protective agent in plant cell cultures. However, it could not be clearified whether this protein-protective property is due to a specific protection against proteases or an unspecific osmotic stabillization effect making the aquous medium environment more comfortable for proteins – or both.

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