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# A downstream process allowing the efficient isolation of a recombinant amphiphilic protein from tobacco leaves

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ABSTRACT

The 65-kDa isoform of human glutamic acid decarboxylase (hGAD65) is a major autoantigen in autoimmune diabetes. The heterologous production of hGAD65 for diagnostic and therapeutic applications is hampered by low upstream productivity and the absence of a robust and efficient downstream process for product isolation. A tobacco-based platform has been developed for the production of an enzymatically-inactive form of the protein (hGAD65mut), but standard downstream processing strategies for plant-derived recombinant proteins cannot be used in this case because the product is amphiphilic. We therefore evaluated different extraction buffers and an aqueous micellar two-phase system (AMTPS) to optimize the isolation and purification of hGAD65mut from plants. We identified the extraction conditions offering the greatest selectivity for hGAD65mut over native tobacco proteins using a complex experimental design approach. Under our optimized conditions, the most efficient initial extraction and partial purification strategy achieved an overall hGAD65mut yield of 92.5% with a purification factor of 12.3 and a concentration factor of 23.8. The process also removed a significant quantity of phenols, which are major contaminants present in tobacco tissue. This is the first report describing the use of AMTPS for the partial purification of an amphiphilic recombinant protein from plant tissues and our findings could also provide a working model for the initial recovery and partial purification of hydrophobic recombinant proteins from transgenic tobacco plants.



## 1. Introduction

The use of platforms based on plants for the production of valuable recombinant proteins has moved from a proof of principles to a competitive commercial process over the last 30 years, with the support of the plant biotechnology and pharmaceutical communities [1]. Plants have several advantages over traditional platforms for recombinant protein production including the economy and scalability of upstream production, and the intrinsic safety benefits of cells that do not support the replication of human pathogens [2].

We previously explored the use of plants for the production of one of the major autoimmune diabetes autoantigens, i.e. the 65-kDa isoform of human glutamic acid decarboxylase (hGAD65), a complex molecule containing 15 cysteine residues and two palmitoylated sites [3]. Recombinant hGAD65 plays a key role in the diagnosis of autoimmune diabetes and is also being investigated in clinical trials as a potential preventative and therapeutic. Several platforms were tested for the recombinant expression of the protein as reviewed in [4]. When expressed in tobacco plants, the protein accumulates to only low levels ($10.5 \pm 2.6$ μg/g fresh leaf weight (FLW); [5]) and is associated with the chloroplast and mitochondrial membranes. We engineered an enzymatically-inactive mutant (hGAD65mut) that accumulates to 10-fold higher levels than the wild-type enzyme, suggesting that the greater yields of the mutant version may reflect the ability of the wild-type enzyme to disrupt metabolic processes in the plant cell. Although hGAD65mut lacks catalytic activity, it retains its immunoreactivity and therefore remains suitable for diagnostic and therapeutic applications [6].

Interestingly, highest hGAD65mut levels were obtained in stable transformed tobacco plants when compared e.g. to transiently transformed *Nicotiana benthamiana* leaves. We also investigated different sub-cellular targeting strategies, eventually selecting the membrane-anchored mutated form of hGAD65mut.

Tobacco has several advantages as an expression host including its prolific biomass production per hectare (resulting in high overall product yields) and its status as a non-food/feed crop, which reduces the risk of adventitious contamination of the food/feed

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chains. The selected tobacco line was bred to homozygosity to establish genetic and phenotypic stability, which is a prerequisite for the development of a master seed bank as part of a GMP-compliant production process (EMEA, 2008). The yields of hGAD65mut were evaluated by radioimmunoassay after eight generations of self-pollination, achieving a productivity of up to 106.3 μg/g FLW. Such yields represent a promising starting point for the development of an efficient and robust downstream process for protein extraction and purification.

The purification of GAD65 from microbial, yeast and animal sources typically involves combinations of affinity, anion exchange and size exclusion chromatography. In the case of plants, however, recombinant proteins typically accumulate inside the cell and the early downstream processing (DSP) steps are more challenging. We established the first DSP steps for transgenic plant material, starting with the release of protein from the biomass by homogenization, and followed by aqueous extraction, preliminary clarification and initial capture/enrichment. We also evaluated these processes in the context of sustainable large-scale manufacturing of hGAD65mut using transgenic tobacco leaves. This evaluation was based on the following parameters: the overall economics of production, the concentration of recombinant immunoreactive hGAD65mut in the final processed extract, the prevention of product aggregation, and the removal of contaminants such as host cell proteins (HCPs) and phenolic compounds. We selected a protocol by evaluating different extraction reagents, a clarification treatment and different aqueous micellar two-phase protocols. The experimental design was based on a combination of protocols previously used for the extraction and purification of hGAD65 from other organisms and for the extraction and purification of diverse proteins from leaf tissues.

## 2. Materials and methods

### 2.1. Plant material

The T8 generation of transgenic tobacco plants (*Nicotiana tabacum*, cv. Petit Havana SR1) was produced by self-crossing the best-performing T1 hGAD65mut transgenic plant and repeating the procedure over seven further generations, checking the performance in each generation by radioimmunoassay.

Plants were cultivated in a greenhouse at 25/22 °C day/night temperature with a 16-h photoperiod at 70% relative humidity. The plants were grown for 60 days prior to harvest.

### 2.2. Reagents and chemicals

All chemicals and reagents were analytical grade and were purchased from commercial sources. Triton X-100, Triton X-114, Tween-20 and pentaethylene glycol monododecyl ether detergents, and glycerol, polyethylene glycol (PEG) with an average molecular weight of 20,000 g/mol, NaCl and $(NH_4)_2SO_4$ were supplied by Sigma-Aldrich (St. Louis, MO, USA). Nonidet P-40 (NP-40) was obtained from Fluka (St. Louis, MO, USA) and technical-grade dextran with a molecular weight of 500,000 g/mol (dextran T500) was obtained from Carl Roth (Karlsruhe, Germany).

### 2.3. Filtration tools

Miracloth membrane was supplied by Calbiochem (Merck KGaA, Darmstadt, Germany) and Supracap™ Depth Filter Capsules were manufactured under a certified quality management system by Pall Corporation (Port Washington, NY, USA).

### 2.4. Aqueous micellar two-phase system

The aqueous micellar two-phase systems (AMTPS) were prepared on a w/v% or w/w% basis by mixing the required quantities of phase-forming solutes with the crude hGAD65mut extract. We prepared 5-g or 5-ml AMTPS samples by gentle mixing to achieve equilibration, then separation was promoted by incubating at a specific temperature for 10 min before complete phase separation by low-speed batch centrifugation at 4000 × *g* for 10 min. The top and bottom phase volumes were determined using graduated tubes allowing the experimental volume ratio ($V_t/V_b$) to be estimated. Individual top and bottom phases and potential pellets or interphases were collected carefully for further analysis.

### 2.5. Gel electrophoresis and Western blots

The protein samples were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% (w/v) polyacrylamide gels and post-stained with Coomassie Brilliant Blue R-250 for the analysis of the total soluble protein (TSP), including the recombinant target protein and plant proteins.

For western blot analysis, proteins in unstained gels were electroblotted onto a nitrocellulose membrane, and hGAD65mut was detected using a rabbit anti-GAD65/67 G5163 (IgG) polyclonal antibody (Sigma-Aldrich), diluted 1:5000. This recognizes a linear epitope at the C-terminus of human GAD65 and GAD67. The bound antibody was detected with a secondary peroxidase-conjugated goat anti-rabbit IgG (whole molecule) A6154 (Sigma-Aldrich) diluted 1:5000, and the signal was revealed by staining with Amersham ECL Select (GE Healthcare, Fairfield, USA) or DAB Peroxidase substrate (Sigma FAST™ 3,3′-diaminobenzidine tetrahydrochloride with metal enhancer).

### 2.6. Quantification of total proteins and hGAD65mut

The TSP content was measured by using the *ortho*-phthalaldehyde/*N*-acetyl-L-cysteine (OPA/NAC) reagent [7]. Briefly, a 10-μl sample was mixed with 300 μl of the working reagent and the absorbance was measured at 335 nm. All assays were conducted in triplicate, with solvents for blank corrections and bovine serum albumin (BSA) as a standard. Averages were used in the calculations.

The concentration of hGAD65mut in each sample was determined by radioimmunoassay using hGAD65 autoantibody-positive serum from a T1D patient and $^{125}$I-GAD65 (RSR, Cardiff, UK) as a tracer [8]. Commercial recombinant human GAD65 (rhGAD65) produced in the baculovirus expression system (Diamyd, Karlavagen, SE) was used as positive control. Untransformed control plants were analyzed to exclude any potential negative effects of buffer and host components in the detection procedure.

### 2.7. Quantification of phenolic compounds

The total phenolic content of the tobacco extracts was determined as previous described [63]. The samples were diluted to two different concentrations with distilled water (final volume 300 μl) and mixed with 150 μl of Folin–Ciocalteu reagent. The mixture was allowed to stand for 1 min at room temperature before adding 600 μl of 20% (w/v) $Na_2CO_3$ solution and mixing extensively. The reaction was incubated for a further 1.5 h at room temperature before the absorbance was measured at 725 nm. The phenolic content was determined against gallic acid standards (concentration range 0–50 μg/ml).