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Optimization of the heterologous expression of banana glucanase in *Escherichia coli*

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Abstract: For the heterologous production of a banana glucanase in *Escherichia coli*, its gene (GenBank GQ268963) was cloned into a pG EX-4T expression vector as a fusion protein wit h glutathione-*S*-transferase (GST). BL21 cells transformed with the GST-Mus a 5 con struct were employed for production of the protein induced by 1 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG). The conditions for protein expression were opti mized by varying the tem perature (25, 30 and 37 °C) and duration of protein expression (3, 6 and 12 h). The level of protein production was analyzed by densitometry of the sodium dodecyl sulfate–polyacrylamide gel (SDS–PAG) after electrophoretic resolution of the respective cell lysates. The optimal protein expression for downstream processing was obtained after 12 h of cell growth at 25 °C upon addition of IPTG. Recombinant GST-Mus a 5 purified by glutathione affinity chromatography revealed a molecular mass of a bout 60 kDa. The IgE and IgG reactivity of the rGST-Mus a 5 was confirmed by dot blot an alysis with sera of individual patients from subjects with banana allergy and polyclonal rabbit antibodies against banana extract, respectively. The purified reco mbinant glucanase is a potential candidate for banana allergy diagnosis.

Keywords: food allergen; protein expression; glucanase.

INTRODUCTION

Immunoglobulin E (IgE)-mediated allergy affects more that 25 % of the world's population, and b elongs to the most chronic disorders in m odern society.[1] The key issue for the treat ment of allergies is the em ployment of reliable diagnostic reagents. The allergen extracts currently employed for diagnosis re-

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present mixtures of allergens and non-allergenic material. Diagnostic tests for food allergy frequently have poor specificity and sensitivity,[2] as the quality of allergen extracts from fruits and other plant-derived foods can vary due to the inherent presence of proteolytic enzymes, the ripening stage and/or storage conditions of the allergenic source materials.[3,4] Therefore, replacement of allergen extracts with a panel of IgE reactive molecules from an allergen source is a promising strategy for the improvement of allergy diagnostics. A panel of three cherry recombinant allergens was superior to diagnostic methods based on cherry extract.[5] In this respect, the evaluation of allergenic properties of a particular allergen candidate for component-resolved diagnostics needs to be performed.

Allergy to banana fruit has been reported as an isolated allergy, but sometimes it is associated with pollen or latex allergy.[6] Component-resolved diagnostics (CRD) of allergy to plant foods is essential for the clinical management of allergic patients.[7] Specific IgE immunodetection has enabled the identification of several IgE binding components in banana fruit, covering a wide range of molecular sizes. However, putative allergens of 30–37 kDa are the most frequently recognized in the sera from allergic patients.[8–10] The molecular basis of banana allergy has been ascribed to five IUIS (www.allergen.org) nominated allergens: profilin (Mus a 1), class I chitinase (Mus a 2), non-specific lipid transfer protein (Mus a 3), thaumatin-like protein (Mus a 4) and beta-1,3-glucanase (Mus a 5).

Most fruit allergens are structurally and evolutionary related to "pathogenesis-related proteins" (PR-proteins).[11] Beta-1,3-glucanase belongs to the PR-2 family of proteins and is involved not only in plant defense, but also in diverse physiological and developmental processes. Besides banana fruit,[12] IgE reactive beta-1,3-glucanases were found in several pollens (olive, ash and birch), vegetables (tomato, potato and bell pepper) and latex.[13,14] Palomares *et al.* showed that beta-1,3-glucanases contribute to the latex–pollen–vegetable food cross-reactivity,[14] In addition, an occupational allergy due to Ole e 9, glucanase from olive pollen has been reported.[15] The crystal structure of the banana glucanase has been revealed and its three-dimensional structure exhibits a canonical $(\beta/\alpha)_8$ TIM-barrel motif found in other glucan endohydrolases.[16]

The aim of this work was to optimize the procedure for recombinant banana glucanase (Mus a 5) production in *E. coli* and its downstream processing for potential application in component-resolved allergy diagnostics.

EXPERIMENTAL

Bacterial strain and construct

E. coli BL21-CodonPlus (DE3)-RIPL cells, kindly provided by Dr Knud Poulsen (University of Aarhus, Denmark), were transformed with the construct pGEX-4T-glucanase denoted as rGST-Mus a 5. The cloning strategy was to produce recombinant Mus a 5 (GenBank GQ268963) with glutathione-*S*-transferase as an expression tag on the *N*-terminal. Total RNA was isolated from banana fruit by an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany)

according to the manufacturer's instructions. Cyclic-DNA (cDNA) was transcribed by a RevertAid^{TM} First Strand cDNA synthesis kit (Fermentas UAB, Vilnius, Lithuania). For amplification of the mature gene of Musa 5, sense and antisense – specific primers with EcoR I and Xho I restriction sites (marked in italic) 5'-GAATTCATTGGTGTCTGCTACGG-3' and 5'-CTCGAGCTAAAAGCTTATTTGGTAGAC-3' were used, respectively. The amplified Musa 5-encoding fragment, was cloned into a pGEX-4T vector. The construct was verified by DNA sequencing.

Cell growth and induction of protein expression

Inocula were prepared from transformed BL21 cells that were grown overnight at 37 °C in LB medium (1 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl, 2 g L^{-1} glucose) containing 100 mg L^{-1} ampicillin, 25 mg L^{-1} chloramphenicol and 25 mg L^{-1} kanamycin. The culture (0.5 mL) was introduced into 10 mL of LB medium containing the respective antibiotics. Once the absorbance (OD_{600}) reached a value of 0.6 following an initial growth phase, protein expression was induced with 1 mM IPTG (Fermentas), and the cells were grown at 25, 30 or 37 °C. Following induction of protein expression, aliquots (1 mL) were taken after 3, 6 and 12 h.

SDS–PAGE analysis

The GST-Musa 5 protein expression was analyzed in cell lysates of induced and non-induced bacteria by SDS–PAG electrophoresis under reducing conditions, as outlined by Laemmli.[17] In brief, prior to separation on 14 % SDS–PAG, cell lysates (100 μg) were incubated in sample buffer (6 mM Tris–HCl, 5 % 2-mercaptoethanol, 2 % SDS and 25 % glycerol) for 5 min at 95 °C and centrifuged (14000×g, 1 min). After electrophoresis, the proteins in the gel were stained with Coomassie brilliant blue (CBB-R250, Serva, Germany). For comparison of the level of rGST-Musa 5 protein expression under different experimental conditions, the SDS–PAG was scanned for densitometric quantification using GelPro Analyzer 3.1 (Media Cybernetics).

Isolation of rGST-Musa 5

Recombinant GST-Musa 5 was purified from a BL21 cell culture (100 mL), after 12 h of protein expression at 25 °C. The cells were harvested by centrifugation (3000×*g*, 15 min), and the pellet was suspended in 25 mL of ice-cold L buffer (5 mM Tris–HCl, pH 8.0, 10 mM NaCl, 5 mM EDTA, 0.1 % NaN_3 and 0.1 % Na-deoxycholate). Immediately before use, PMSF (0.1 mM) and DTT (1 mM) were added to the L buffer. After sonication (3×20 s, 20 rms, Branson Sonifier 150), $MgSO_4$ (1 mM), benzonase (0.01 mg mL^{-1}, Novagen) and lysozyme (0.1 mg mL^{-1}, Serva, Germany) were added to the cell lysate, which was further incubated at RT for 15 min. To collect the insoluble fraction (IF), the cell lysate was centrifuged (3000×*g*, for 15 min). After two washings with the buffer (5 mM Tris–HCl, pH 8.0, 10 mM NaCl, 5 mM EDTA, 0.1 % NaN_3), the IF was solubilized in S buffer (10 mM Tris, 5 mM glycine, 6 M urea, pH 8.0). Protein refolding was achieved by rapid mixing of denatured protein solution with R buffer (30 mM NaCl, 2 mM Na_2HPO_4, 3.6 mM KH_2PO_4, pH 7.50; 1:7, v:v), in which a cocktail of protease inhibitors (1 mL L^{-1} of buffer) and oxidized (0.3 mM GSSG) and reduced glutathione (3 mM GSH) were added. The rGST-Musa 5 solution was applied onto a pre-equilibrated GST-Bind® resin (Novagen) according to the manufacturer's instruction.[18] The concentration of the rGST-Musa 5 protein was determined using a molar extinction coefficient of 1.434, which was calculated from the amino acid sequence by ProtParam (http://expasy.org/cgi-bin/protparam).

Production of the polyclonal antibodies to banana extract

Two rabbits were immunized according to the protocol described by Harlow and Lane.[19] In brief, 0.25 mL of banana extract (0.5 mg mL^{-1}) which was prepared according to Gavrović-Jankulović *et al.*[20] was mixed with 0.25 mL CFA (complete Freunds adjuvant) for the first immunization. Every 15 days, for four months, the rabbits were boosted with a mixture of 0.25 mL of the banana extract and 0.25 mL of IFA (incomplete Freunds adjuvant). Each rabbit was subcutaneously immunized with 0.5 mL of the emulsion. After four months, sera were collected and the antibodies were pooled and fractionated by ammonium sulfate (50% saturation). After dialysis against phosphate buffer saline (PBS) antibodies were aliquoted and stored in 20% glycerol at –20°C.

Dot blot

Six sera from persons with positive clinical history to banana allergy and positive skin prick test to banana extract were used for the evaluation of IgE reactivity of rGST-Mus a 5 in dot blot. A pool of three sera from persons without banana allergy was used as a control. Purified rGST-Mus a 5 (5 μg) was applied to a nitrocellulose membrane (NC) using 96-well dot blot hybridization manifold (VWR, Vienna, Austria). To discriminate IgE reactivity of the GST tag from IgE reactivity of glucanase, rGST was applied onto the NC membrane as a control. Membranes were blocked using 30 mM Tris buffer saline (TBS) pH 7.4, containing 5% w/v skimmed milk for 1 h at RT. Membranes were incubated with the pooled sera in 1 % skimmed milk in TBS (dilution, 1:3, v:v) overnight at RT. Goat anti-human IgE (dilution 1:10000, v:v) was used for detection of IgE binding. Alkaline phosphatase-labeled rabbit anti-goat IgE antibodies (dilution 1: 30000, v:v, Sigma) were used as the tertiary antibody. IgE reactive spots were visualized with BCIP/NBT solution.

For detection of IgG reactivity of rGST-Mus a 5, rabbit antibodies against banana extract were employed. After blocking, the membrane was incubated with the primary antibodies (dilution 1:5000) for 2 h at room temperature. After three washings, the membrane was incubated with alkaline phosphatase anti-rabbit antibodies (dilution 1:30000, Sigma–Aldrich, Missouri, USA) as previously described.[21]

IgG inhibition assay

For IgG inhibition assays natural Mus a 5[22] (5 μg) was applied to the NC membrane according to previously described procedure. After blocking, the respective membranes were incubated overnight with anti-banana rabbit antibodies, which had been previously incubated for 2 h with two different concentrations of rGST-Mus a 5 (5 and 50 μg mL^{-1}).

RESULTS AND DISCUSSION

Optimization of rGST-Mus a 5 expression

Structural homogeneity, batch-to-batch consistency and unlimited availability makes recombinantly produced proteins advantageous over their natural counterparts. Various expression systems have been adopted for recombinant protein production. Natural banana glucanase is a 32 kDa protein that possesses no post-translational modifications,[12] making prokaryotic cells a suitable option for its expression. Protein purification with a glutathione-*S*-transferase (GST) affinity tag, which was introduced in 1988 by Smith and Johnson,[23] is based on the strong affinity of GST for glutathione-covered matrices. In the GST gene fu-

sion system, expression is under the control of the *tac* promoter, which is induced using the lactose analogue IPTG. Induced cultures are allowed to express GST fusion proteins for several hours before the cells are harvested. *E. coli* BL21 is a protease-deficient strain specifically selected to give high efficiency transformation and high level of expression of GST fusion proteins.[24]

The cloning strategy of Mus a 5 into the pGEX expression vector is given in Fig. 1. For the optimization of the expression of a given protein construct, a time-course analysis of the level of protein expression in recommended.[25] Therefore, for the optimization of rGST-Mus a 5 expression upon induction of protein synthesis, BL21 (DE3) cells were grown at different temperatures, *i.e.*, 25, 30 and 37 °C. Aliquots taken after 3, 6 and 12 h upon rGST-Mus a 5 induction were analyzed by SDS–PAG electrophoresis. Densitometric comparison of the intensity of the rGST-Mus a 5 band with all other constitutively expressed proteins per line, revealed that the highest yield of expression was obtained after 12 h of induction of protein synthesis under all the tested temperatures (Fig. 2). The band of about

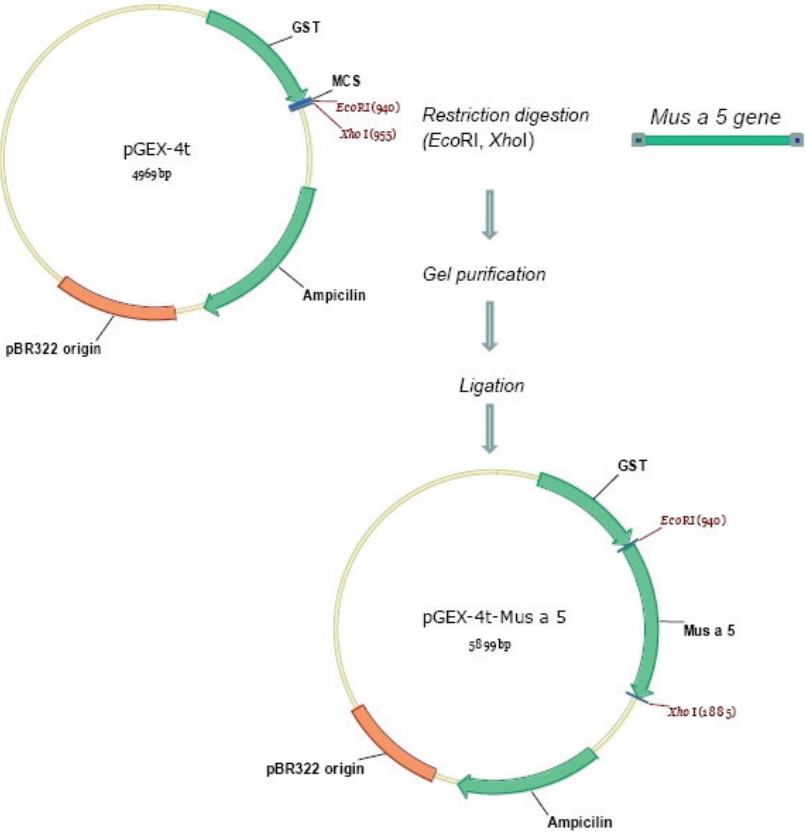

Fig. 1. Cloning of banana glucanase gene into the pGEX-4T vector.

60 kDa, representing rGST-Mus a 5, is predominant in all tested samples, except in the non-induced cells with the pGEX-4T-glucanase plasmid (Fig. 2, lane 1) and after 3 h of induction of protein synthesis at 25 °C (Fig. 2, lane 2). Taking into consideration the presence of other proteins in the cell lysates, the optimal conditions for the protein expression and further downstream purification was protein synthesis for 12 h at 25 °C.

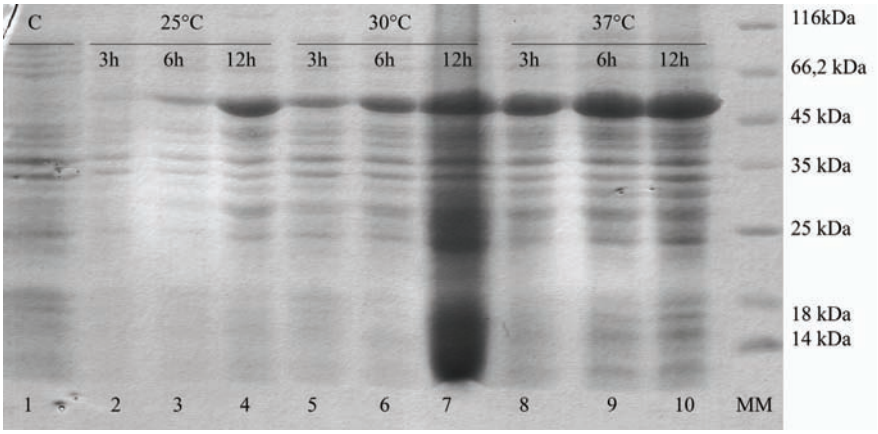

Fig. 2. Time course of rGST-Mus a 5 expression at 25, 30 and 37 °C. Expression of rGST-Mus a 5 was induced with 1 mM IPTG. Aliquots were removed at the times indicated. Proteins were visualized by Coomassie brilliant blue staining; line 1: control, MM: molecular markers.

Isolation of rGST-Mus a 5

Recombinant GST-Mus a 5 was isolated from BL21 cells by glutathione affinity chromatography. Prior to affinity purification, the cells were harvested by centrifugation and after resuspension were lysed with lysozyme. Benzonase was employed to reduce the viscosity of the cell lysate caused by nucleic acids. Further SDS–PAG electrophoresis revealed that rGST-Mus a 5 was accumulated in the insoluble fraction of the cell lysate, and its solubilization was achieved with 6 M urea. Prior to separation, denatured rGST-Mus a 5 was refolded by rapid batch dilution and subsequently applied onto an affinity column. The homogeneity of the isolated rGST-Mus a 5 was assessed by SDS-PAG electrophoresis, revealing a protein band of about 60 kDa (Fig. 3). The yield of the purified rGST-Mus a 5 was about 35 mg L^{-1} of LB, as calculated using the molar extinction coefficient for rGST-Mus a 5.

IgE and IgG reactivity of rGST-Mus a 5

The IgE reactivity of the rGST-Mus a 5 was examined by dot blot analysis using the sera of six banana allergic patients. To evaluate the IgE reactivity of the fusion tag, recombinant GST was also tested as a control. IgE reactivity was

detected only for the rGST-Mus a 5 (Fig. 4), while no IgE binding was found for GST. Although the correct protein folding should be confirmed by a thorough structural characterization, the IgE reactivity of the rGST-Mus a 5 suggests that this protein could find application as a diagnostic reagent in banana allergy.

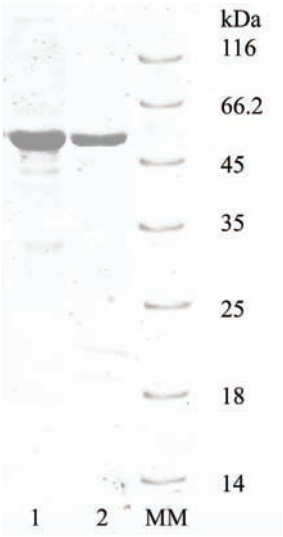

Fig. 3. SDS-PAG electrophoresis of purified rGST-Mus a 5 produced in *E. coli*: 1 – solubilized rGST-Mus a 5 fraction; 2 – rGST-Mus a 5 eluted from the affinity matrix; MM – molecular markers.

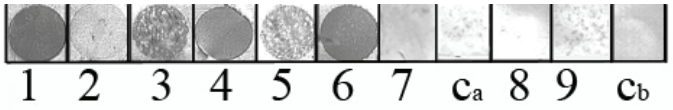

Fig. 4. IgE reactivity of rGST-Mus a 5: 1–6 – individual sera from banana allergic persons, 7 – pool of sera from 3 healthy individuals, c_a – control of secondary antibody; IgE reactivity of rGST: 8 – pool of sera from 6 banana allergic persons, 9 – pool of sera from 3 healthy individuals and c_b – control of secondary antibody.

The IgG reactivity of rGST-Mus a 5 was shown by dot blot analysis with anti-banana rabbit antibodies (Fig. 5).

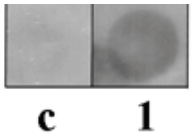

Fig. 5. IgG reactivity of rGST-Mus a 5: c – control, 1 – rGST-Mus a 5.

IgG inhibition assay

To evaluate the IgG reactivity of the rGST-Mus a 5, an inhibition assay with anti-banana rabbit antibodies was performed. Compared to the positive control, IgG binding to natural Mus a 5 was reduced with 5 μg of rGST-Mus a 5. Complete inhibition was achieved by pre-incubation of the antibodies with 50 μg of

inhibitor (Fig. 6), suggesting that the rGST-Mus a 5 shares immunodominant epitopes with natural Mus a 5.

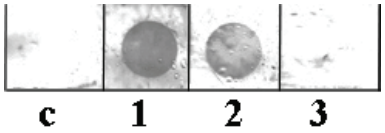

Fig. 6. Inhibition of IgG binding to natural Mus a 5 by rGST-Mus a 5. The membranes were incubated with c – secondary antibodies, 1 – anti-banana antibodies, 2 – anti-banana antibodies pre-incubated with 5 μg rGST-Mus a 5, 3 – anti-banana antibodies pre-incu–bated with 50 μg rGST-Mus a 5.

CONCLUSIONS

An optimization of expression of recombinant banana glucanase - Mus a 5 in a prokaryotic expression system with a GST fusion tag is reported. Optimal expression of rGST-Mus a 5 in *E. coli* BL21(DE3) cells was established (12 h protein expression at 25 °C), and the protein was isolated by glutathione affinity chromatography. The rGST-Mus a 5 showed IgE reactivity with five out of six individual sera of banana allergic subjects. A dot blot inhibition assay with anti--banana rabbit antibodies revealed that the rGST-Mus a 5 shares immunodominant epitopes with natural Mus a 5. Further structural and immunological characterization should assess the applicability of the recombinant banana glucanase for the diagnosis of banana allergy.

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ИЗВОД

ОПТИМИЗАЦИЈА ХЕТЕРОЛОГЕ ПРОИЗВОДЊЕ ГЛУКАНАЗЕ ИЗ БАНАНЕ У *E. coli*

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За потребе производње у *Escherichia coli* ген глуканазе из банане (GenBank GQ268963) је уклониран у експресиони вектор pGEX-4T са глутатион-*S*-трансферазом (GST). Производња овог протеина у ћелијама је индукована 1 mM изопропил-*β*-D-тиогалактопиранозидом (IPTG). Услови за експресију протеина су оптимизовани варирањем температуре (25, 30 и 37 °C) и дужине трајања протеинске синтезе (3, 6 и 12 h). Ниво производње протеина је анализиран денситометријом SDS–PA гела након електрофоретског раздвајања ћелијских лизата. Оптимална производња протеина за његово даље процесовање је добијена гајењем ћелија након додатка IPTG на 25 °C током 12 h. Рекомбинантни GST-Mus a 5 пречишћен афинитетном хроматографијом са глутатионом показује молекулску масу од 60 kDa. IgE и IgG реактивност изоловане глуканазе потврђена је у "dot blot" са појединачним серумима особа алергичних на банану, и са поликлонским зечијим антителима на екстракт банане, редом. Пречишћена рекомбинантна глуканаза је потенцијалан кандидат за дијагнозу алергије на банану.

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