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Expression and Thrombin Cleavage of *Poa p* IX Recombinant Allergens Fused to Glutathione S-Transferase

Key Words

Expression cloning
Recombinant allergens
pGEX
Thrombin cleavage
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Frame shift
pGEX 2T-1
Glutathione-S-transferase

Abstract

The high-level expression and purification of *Poa p* IX recombinant grass pollen allergens were examined utilizing a modified pGEX plasmid, designated as pGEX 2T-1. This vector permits frame-1 ligation of λ gt11 cDNA inserts and cleavage of the recombinant allergenic protein from the fusion partner glutathione S-transferase. The expression of the fusion proteins in water-soluble form varied among the transformants of the same bacterial strain and also between different host strains. Purification of the fusion proteins by affinity chromatography employing glutathione agarose gel revealed that proteases in the bacterial lysate bound to the gel and were co-eluted with the fusion proteins. These proteases, which specifically degraded the recombinant proteins to varying degrees, were inhibited by both of the inhibitors, phenylmethylsulfonyl fluoride and aprotinin. Cleavage by thrombin of the fusion proteins indicated that the structure of the individual protein affected the thrombin accessibility to the cleavage site. Increased concentration of thrombin partly compensated this effect, but resulted in a broader specificity of the enzyme. By contrast, cleavage of the fusion protein when it was still attached to the glutathione gel was convenient and led to purification of the product devoid of proteolytic activity. Since almost all the recombinant allergens have been cloned in λ gt11 vector, the pGEX 2T-1 vector reported herein will facilitate the synthesis, purification of the corresponding allergenic proteins or their peptides in soluble and biologically active forms.

Introduction

Recently molecular cloning and sequencing of a number of allergenic proteins have led to the increase in knowledge of their primary structures [1-4]. However, the clinical use of these recombinant allergens have been limited since the available expression cloning systems do not permit the synthesis of these allergens in water-soluble

and undegraded forms [5-7]. The foreign proteins are often expressed in *Escherichia coli* as fusion proteins (FPs); this in-frame fusion with a bacterial protein presumably protects the recombinant protein from degradation by bacterial proteases [5, 8, 9]. However, there are several difficulties associated with the synthesis and purification of FPs: (1) they are frequently localized within insoluble bodies in the bacterial cells [8], although the constituent com-

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ponents of the FP may be soluble in their native state, (2) the denaturing agents required for solubilization and further purification of the FPs may affect the conformation and biological activity of the recombinant molecules, and (3) the fusion partner or cleaving agent which may contaminate the recombinant protein can restrict further use of the latter.

The pGEX vectors permit expression of the cloned DNA fused to glutathione S-transferase (GST) gene, and enable the synthesis of the FPs in water-soluble form [10]. These vectors exist as pGEX-1, pGEX-2T and pGEX-3X, representing reading frame-1, frame-2, and frame-3, respectively. The latter two of these also encode sites at the C-terminus of GST which allow proteolytic cleavage of the FP by thrombin and factor Xa, respectively, and facilitate the purification of recombinant proteins. Moreover, FPs can be specifically purified from the bacterial extract by glutathione-agarose gel affinity chromatography by a one step procedure [10, 11]. However, extensive use of the pGEX vectors [10] for expression and purification of cloned allergens is limited by (1) the lack of a cleavage site in pGEX-1, which constitutes the most suitable vector for in-frame expression of λ gt11 inserts, (2) the variable efficiency of cleavage by thrombin, and (3) degradation of the recombinant protein by contaminating proteases, often resulting in low yield of recombinant protein.

Previously we investigated the immunological properties of recombinant grass pollen allergens employing the pWR 590-1 vector, which led to the expression of the allergenic molecules as water-insoluble FPs [4, 12, 13]. In this study we report the modification of the vector pGEX-2T for the expression of the cDNA inserts in frame-1. Furthermore, we have optimized the purification and cleavage of the FPs, which allows rapid isolation of water-soluble, recombinant allergenic polypeptides.

Materials and Methods

Enzymes and Inhibitors

Thrombin, phenylmethylsulfonyl fluoride (PMSF) and aprotinin were purchased from Sigma Chemicals Co., St. Louis, Mo.

Modification of the Plasmid pGEX-2T

The plasmid pGEX-2T (Pharmacia, Montreal, Canada) which contain a multiple cloning site consisting of BamHI, SmaI and EcoRI, was cleaved by SmaI and ligated to the EcoRI-linker 5'GGAATTCC (Pharmacia, Montreal). After digestion by EcoRI, the vector was ligated at 13°C overnight and the ligation products were transformed into *E. coli* JM105. Ampicillin-resistant clones which contained plasmid cleavable by EcoRI but resistant to SmaI were selected. This modified plasmid was designated as pGEX 2T-1.

Cloning and Expression of KBG3.1 and KBG8.3 Inserts

Two clones KBG3.1 and KBG8.3 were isolated from a λ gt11 cDNA library corresponding to the mRNA of Kentucky bluegrass pollen [4]. The DNAs of these clones were isolated, digested with EcoRI and inserts were purified as described elsewhere [4]. Two λ gt11 cDNA clones, KBG3.1 and KBG8.3 respectively, encode the N- and the C-terminal fragments of the *Poa p* IX allergens of Kentucky bluegrass pollen [4, 13]. The inserts of these two λ clones were ligated to the pGEX 2T-1 vector and transformed into *E. coli* strain JM105, TG1- λ or MV1193. Transformants expressing IgE-binding FPs were selected as described elsewhere [4].

Purification of FPs

Recombinant proteins and GST from the self-ligated vector were isolated by appropriate modification of the original protocol [10]. Briefly, overnight cultures were diluted 1 in 10 into 1 liter of L-broth containing 50 μ g/ml of ampicillin and incubated at 37°C for 1–4 h before addition of isopropyl thiogalactopyranoside at 0.5 mM to induce expression. After 1–4 h of incubation, cells were pelleted, resuspended in 20 ml of 0.2 M Na-phosphate buffer, pH 7.3, 0.15 M NaCl (HPBS) containing 1% Triton X-100 and quick-frozen at –70°C. The cells were thawed at 37°C, sonicated and the lysates were cleared by centrifugation at 18,000 g for 20 min at 4°C. Approximately 20 ml of the supernatants were mixed with swollen and washed glutathione agarose beads (Sulphur Linkage, Sigma Chemical Co., St. Louis, Mo.) in the ratio 10:1 (v:v) to allow specific binding of the GST moiety of the FPs to the glutathione on the beads. After incubation on a roller for 30 min at 4°C, the beads were washed 5 times each with 15 ml of HPBS and once with 15 ml of 50 mM Tris-HCl, pH 8.0. The bound material was eluted from the beads with 3 ml of elution buffer/ml of beads containing 50 mM Tris-HCl, pH 8.0 and 5 mM reduced glutathione (Sigma Chemicals Co., St. Louis, Mo.). This step was repeated twice. Eluted materials were aliquoted and stored at –20°C. The yield of the FP ranged from 5 to 10 μ g/ml of bacterial extract.

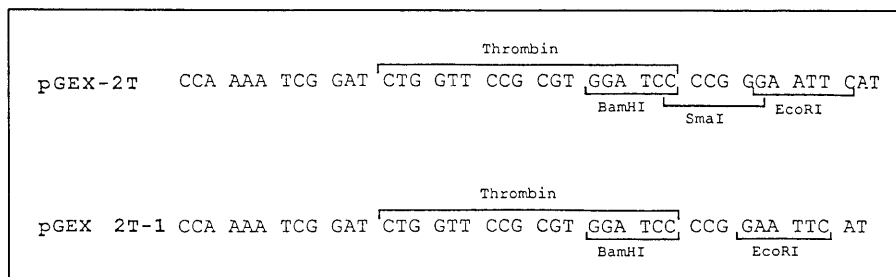
Thrombin Digestion of FPs

To cleave recombinant polypeptides from the GST by thrombin, 150 mM NaCl and 2.5 mM CaCl₂ were added to the purified FP in 50 mM Tris-HCl, pH 8.0, and then added thrombin (1:500; w/w) at room temperature. Alternatively, the FP was cleaved with thrombin when bound to the glutathione-agarose gel. The washed beads were suspended in 50 mM Tris-HCl, pH 8.0 containing 150 mM NaCl and 2.5 mM CaCl₂. Thrombin was added at room temperature and the cleaved proteins were obtained in the supernatant after centrifugation of the beads.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a discontinuous system according to Laemmli [14] using a Fisher mini slab gel apparatus. Samples were prepared in a loading buffer containing 0.06 M Tris-HCl, pH 6.8, 2% SDS and 5% 2-ME. The proteins were blotted onto nitrocellulose membranes by a PolyBlot (American Bionetics, Calif., USA) using a discontinuous buffer system as described by the manufacturer. The membranes were subsequently blocked by incubation in Tris-buffered saline (TBS)/1% gelatin for at least 6 h, and then incubated overnight with a pool of sera (diluted 1:10), of patients allergic to grass pollens, or with murine antiserum to GST (diluted 1:100) in

Fig. 1. Modification of the plasmid pGEX-2T. The DNA sequence at the junction of the GST gene and the polylinker region of the original pGEX-2T plasmid and the modified version, pGEX 2T-1 is shown.



Tris-buffered saline added 0.05%. Tween 20 (TBST)/0.1% gelatin. Bound antibodies were detected by incubation for 2 h with either alkaline phosphatase-conjugated goat anti-human IgE (Tago, Calif., USA), or alkaline phosphatase-conjugated goat antimouse IgG (Zymed, Calif., USA), 2,000-fold diluted in TBST with 0.1% gelatin. The membranes were washed 5 times, 4 min each, in TBST between each incubation step, and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as enzyme substrate.

Results

Figure 1 shows the sequence of the cleavage and cloning sites of the vectors, pGEX-2T and its frame-shift derivative designated as pGEX 2T-1. This new vector lacks the original SmaI site, but has an EcoRI site in the same reading frame as pGEX-1. The vector pGEX 2T-1 when ligated in frame-1 to cDNA inserts of clones which were isolated from a λ gt11 library led to the expression of the allergenic polypeptides [data not shown]. In this study, the expression of two representative cDNAs, KBG3.1 and KBG8.3, and the purification of their corresponding proteins were further investigated.

To examine the extent of solubility of the FPs in the pGEX system, the plasmid containing the KBG3.1 was transformed into three different strains of *E. coli*, JM105, MV1193 and TG1- λ . Five recombinant colonies from among the transformants of each of the above host strain were analyzed for the expression of the FP. Both MV1193 and TG1- λ expressed FPs which were recovered in the water-insoluble fraction [results not shown], while three out of five of the JM105 colonies synthesized water-soluble products. Figure 2 shows SDS-PAGE electrophoretograms of the colonies producing soluble FP (colony 3–5) and of the colonies producing insoluble FP (colony 1–2). The extent of the expression and solubilization of the FP varied among the strains and among colonies of any particular strain. These results suggest that JM105 cells were the most efficient for the expression of soluble FP.

The FPs were purified by adsorption on agarose glutathione gel and subsequently elution through replace-

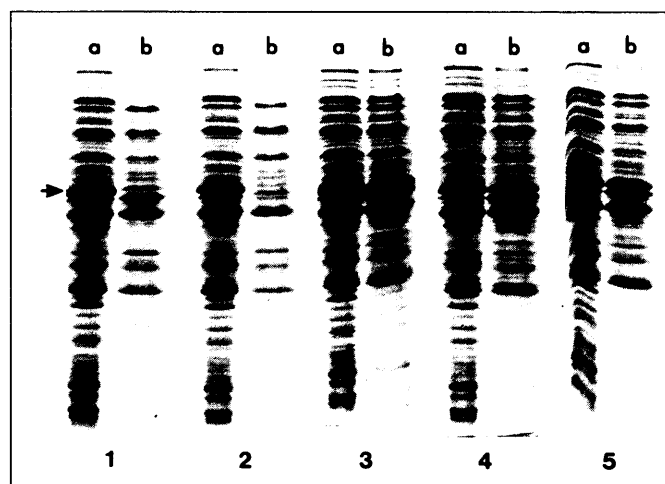


Fig. 2. SDS-PAGE analysis of *E. coli* expressing the FP-rKBG3.1. Overnight cultures of five different colonies, 1–5, were diluted 1/10, grown for 1 h and subsequently induced by isopropyl thiogalactopyranoside for 3 h. Lane a shows sedimented cells added SDS loading buffer; lane b, water-soluble fractions of sedimented cells were prepared as described in Materials and Methods and resuspended in SDS loading buffer to the same cell: final volume ratio as the preparations analyzed in lane a. The arrowhead indicates the position of the FP.

ment by reduced glutathione [10, 11]. This procedure resulted in a high degree of purification, even if the eluted fraction contained traces of other components (fig. 3A, lanes 1 and 2). The purification of GST synthesized by self-ligated control plasmid however lacked these contaminating components indicating that the minor protein bands represented degraded recombinant protein and not other bacterial proteins. Significant degradation of the FP-rKBG8.3 was observed during the dialysis of the eluted FP preparations at 4°C overnight (fig. 3A, lane 3). By contrast, no degradation was seen with the FP-rKBG3.1 or recombinant GST [data not shown]. However, overnight incubation at 37°C of this FP led to significant degradation of the recombinant protein but not GST. These results demonstrate that the eluates contained specific proteo-

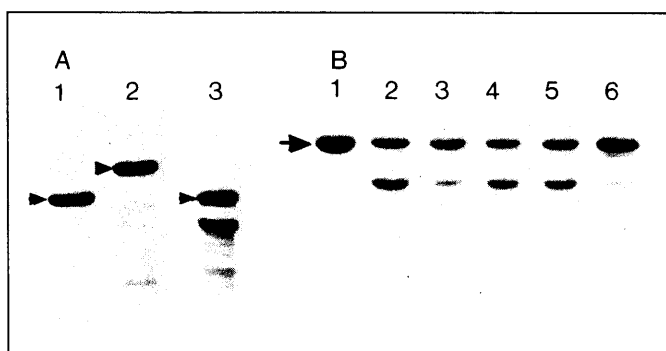


Fig. 3. SDS-PAGE analysis of crude and affinity purified preparations of FP-rKBG8.3 and FP-rKBG3.1. **A** Lane 1, purified FP-rKBG8.3; lane 2, purified FP-rKBG3.1, and lane 3, purified FP-rKBG8.3 after incubation over night at 4°C. The arrows indicate the respective intact fusion proteins. **B** Effect of overnight incubation at room temperature of purified preparations of FP-rKBG8.3 with and without addition of protease inhibitors. Lane 1, FP preparation, and after overnight incubation following no addition (lane 2), addition of 1 mM PMSF (lane 3), 10 kallikrein inhibition unit (KIU) aprotinin (lane 4), 1 KIU aprotinin (lane 5) or 250 KIU aprotinin (lane 6). All samples were analyzed by SDS-PAGE. Arrowheads indicate the position of the intact FPs. The arrow in B indicates the position of FP-rKBG8.3.

lytic activity which caused degradation of the foreign proteins, but not GST.

Attempts to remove the proteolytic activity in the bacterial extract by absorption on CM Affigel blue, which is known to remove proteases from serum [15], were not successful since the FP or GST remained bound to the gel. To prevent proteolytic degradation, the isolated FP was incubated with protease inhibitors, PMSF and aprotinin and both components inhibited the degradation (fig. 3B, lanes 1–6). Addition of the inhibitors to the lysis buffer did not eliminate or reduce degradation of the fusion proteins in the eluates from the glutathione-agarose gel. Since PMSF is an inhibitor of thrombin [16], only aprotinin was examined for its protective role in thrombin cleavage [17].

The two FPs containing rKBG3.1 and rKBG8.3, isolated in eluates from the glutathione gel, showed different susceptibility for cleavage by thrombin (fig. 4). While rKBG8.3 was cleaved from the GST in a dose-dependent manner within 4 h with enzyme substrate ratio of 1:500 (w/w), the FP-rKBG3.1 remained nearly unaffected. Increase in the enzyme ratio to 1:100 resulted in detection of 36, 26, 24 and 12 kilodaltons (kDa) polypeptides as degra-



Fig. 4. SDS-PAGE analysis of fusion proteins after thrombin cleavage (1/500, w/w) in the presence of aprotinin (250 kallikrein inhibition unit/ml). Lanes a–e, FP-rKBG3.1 and lanes f–j, FP-rKBG8.3. Samples were prepared after 0 min (lanes a, f), 30 min (lanes b, g), 90 min (lanes c, h), 4 h (lanes d, i), and overnight (lanes e, j). Lane m represents molecular weight standards. The marks *, **, and ***, respectively, indicate the positions of the intact FP, the cleaved recombinant protein and GST.

dation products of FP-rKBG3.1 (fig. 4). Immunoblotting analysis revealed that in addition to cleavage at the designated site at this concentration, thrombin also cleaved the rKBG3.1 at one internal location [data not shown]. The control GST polypeptide was not digested by thrombin at the high enzyme substrate ratio. These results are taken to indicate that structural properties of the recombinant protein affect the accessibility of thrombin to the cleavage site of the FPs.

The possibility of cleavage of the FP when it is bound to the agarose-glutathione gel and the effect of aprotinin were also examined. The FP-rKBG3.1 was as resistant to cleavage on the gel as in solution [result not shown], while the FP-rKBG8.3 was readily cleavable as shown in figure 5. Only one polypeptide, with the expected molecular size of 16 kDa was detectable in the cleaved product of FP-rKBG8.3 from the gel. The presence of aprotinin during thrombin cleavage resulted in the same amount of eluted protein according to SDS-PAGE analysis. There was no detectable degradation after further incubation for 40 h at room temperature of the cleaved product, even in the absence of aprotinin. These results indicate that no or limited amount of proteolytic activity was present in the eluate after thrombin cleavage directly from the gel.

Discussion

The major findings of this study comprised (1) an increase in repertoire of the pGEX vector plasmids by incorporating an additional vector, and (2) optimization of the expression and cleavage of the recombinant proteins using this vector. The vector pGEX 2T-1 reported in this study facilitates (1) the high-level expression cloning of the cDNAs selected from the λ gt11 library and any other cDNAs in frame-1 by ligating the insert at the EcoRI site; thrombin cleavage of the fusion protein expressed in frame with GST, which was not possible previously, and (2) cloning of the inserts also in frame-2 at the Bam HI site. The results of this study also demonstrated that for an individual recombinant protein, the extent of expression of soluble FP varies not only between host *E. coli* strains but also among the transformant colonies of a particular strain. Although the mechanism underlying this differential expression is not clear, our results indicate that the solubility problem may be resolved by determining the appropriate bacterial host and screening enough transformants.

Our results revealed that proteases in the bacterial lysates bind to the agarose glutathione gel during the isolation

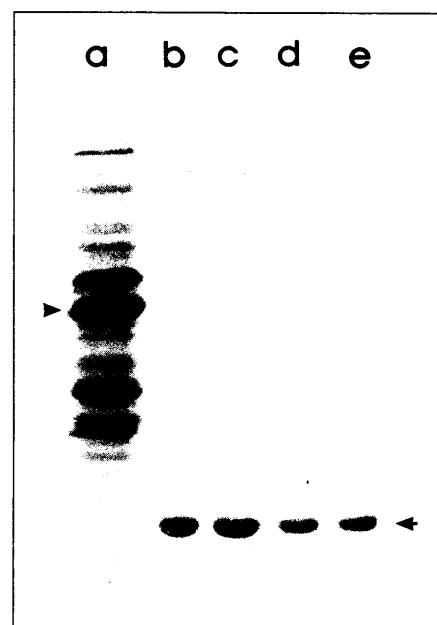


Fig. 5. SDS-PAGE analysis of thrombin cleavage products of FP-rKBG8.3 while attached to agarose glutathione gel. Lane a represents the crude bacterial lysate which was adsorbed to the agarose glutathione gel, lane b the product cleaved by thrombin without aprotinin, lane c the same as in lane b but with aprotinin, lanes d and e the preparations in lanes b and c after overnight incubation at room temperature, respectively. The arrowhead and arrow, respectively, indicate the position of FP and the cleaved protein.

procedure, and are partly desorbed during the elution with glutathione. Interestingly, these enzymes were incapable of degrading GST, whereas recombinant polypeptides were readily degraded. Although the type of proteases involved in degradation of these proteins is unknown, they are most likely serine proteases since their activities were inhibited by both of the serine protease inhibitors PMSF and aprotinin.

PMSF has been shown to inactivate proteases irreversibly by sulfonation of a specific serine residue in the active site of the enzymes [18] and it has been suggested that the inhibition of thrombin activity could be caused by the same mechanism [16]. It is implicit therefore that PMSF may be used initially to inactivate proteases during the lysis of the bacteria. Due to its instability in aqueous solution, it is unlikely to have any inhibitory activity left when thrombin is introduced into the system. Nevertheless, the use of aprotinin is considered to be a safer approach, since it does not affect the activity of thrombin [17]. It is to be noted however that aprotinin, a 6-kDa polypeptide, if used, may be difficult to remove from the eluted FP preparation because of its size.

The direct cleavage by thrombin while the fusion protein still is bound to the glutathione gel was a significant observation since it allowed to avoid the unwanted problem of degradation. It was demonstrated that this procedure was more efficient than that of the cleavage in solution, since it resulted in a product with no detectable protease activity. This finding is in agreement with a recent report on cleavage of GST-FPs [13]. Although this indicated that addition of the inhibitors was unnecessary, it is to be stressed that due to the observed variation in thrombin cleavage of different proteins, this strategy cannot be generalized. Cleaving efficiency by thrombin may be improved by increasing the thrombin concentration, but as is evident from this study, increased thrombin leads to the cleavage within the recombinant protein itself indicating the broadening of specificity of thrombin [18]. Recently the modification of the pGEX-2T vector by introducing a spacer peptide adjacent to the thrombin cleavage site was reported [19] which was shown to increase the accessibility to thrombin and resulted in better cleavage efficiency.

Overall the improvements of the pGEX system reported herein would allow not only synthesis of allergens or their fragments initially cloned in λ gt11 vector in sufficient amounts but also the analysis of their structure-function relationships. The recombinant allergens thus produced may also be used for in vivo studies such as diagnosis of allergies by skin-prick test, however for this purpose, an additional purification step have to be incorporated for the removal of thrombin from the cleaved recombinant allergen.

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