

Production of Recombinant Der fI (a major mite allergen) by *Aspergillus oryzae*

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Der fI is a major mite allergen. To produce Der fI by *Aspergillus oryzae*, we placed a DNA fragment encoding precursor-type recombinant Der fI E(-1)K (reDer fI E(-1)K), which had the C-terminal amino acid of the pro-sequence (Glu) changed to Lys, downstream of the *glaA* gene promoter and introduced it into *Aspergillus oryzae*. In liquid culture, most of the reDer fI E(-1)K produced by the transformants was degraded when culture was shaken vigorously. However, the degradation of reDer fI E(-1)K was suppressed when it was shaken gently. The processed reDer fI E(-1)K could be obtained after lysylendopeptidase and endoglycosidase Hf (Endo Hf) treatment. The yield of processed reDer fI E(-1)K was 8 mg/l. When the transformant was grown on a wheat bran culture, the yield of processed reDer fI E(-1)K reached 48 mg/kg. Because processed reDer fI E(-1)Ks obtained from both cultures had almost the same IgE-binding activity and elicited the same skin reaction as native Der fI, they could be very useful for diagnostic purposes or immunotherapy.

Key words: mite; allergen; fungi; glycosidase

In recent years, the number of patients with allergies such as hay fever, atopic dermatitis, and atopic asthma has been increasing. It is known that the proteinaceous allergens of mites in house dust play a key role in allergic disease. Der fI, a glycoprotein⁶ of the house dust mite *Dermatophagoides farinae*, is one of the major mite allergens. It is known that atopic asthma is triggered by sensitization to Der fI¹ in most cases of patients with atopic dermatitis. It is thought that Der fI is a kind of digestive enzyme, because it has protease activity² and is found in the feces of mites. A method for purification of Der fI from mite cultures has already been established.³ The cDNA encoding Der fI was cloned and from the sequence of Der fI cDNA it is proposed that Der fI is produced as a prepro-form protein comprised of a pre-sequence of 18 amino acids, a pro-sequence of 80 amino acids, and a mature sequence of 223 amino acids.⁴ Investigation of the connection between Der fI and the development of allergic symptoms and attempts to establish desensitization treatment with purified Der fI have been hampered by the lack of available amounts of this protein, because the growth rate of

the mite is very slow.

Extracellular production of reDer fI was first done by a baculovirus expression system.⁵ In this case, reDer fI was produced in the culture as a precursor form. Although the pro-sequence was removed by acid treatment, the N-terminus of acid-treated reDer fI did not become uniform, and its IgE-binding activity of them was lower than that of native Der fI at high protein concentrations.⁵ We made a DNA fragment that was constructed by the pre-sequence and the mature sequence of Der fI cDNA and tried to produce recombinant protein with baculovirus expression system. However, recombinant protein was not recognized in the supernatant by Western blot analysis (unpublished data). We made Der fI E(-1)K DNA, which was constructed by changing the C-terminal Glu codon of the pro-sequence of Der fI cDNA into a Lys codon and produced reDer fI E(-1)K with baculovirus expression system.⁶ Correct processing of the pro-sequence of reDer fI E(-1)K was achieved after lysylendopeptidase treatment. Lysylendopeptidase-treated reDer fI E(-1)K had the same IgE-binding activity as native Der fI. However, it is possible that new antigenicity is produced because the sugar-chains of reDer fI E(-1)K were not completely the same as those of native Der fI.⁶ The sugar-chains of reDer fI were not much trimmed by Endo H or N-glycosidase. For that reason, the production of reDer fI containing the same sugar-chains as those of native Der fI, or no sugar-chain, is important.

Because humans take the allergen directly into their bodies at desensitization treatment, we must be careful to reduce the amount of contaminating substances derived from the host, for example, endotoxin in the case of *Escherichia coli*. The use of an expression system that is safe in every respect is most desirable. *Aspergillus oryzae* (*A. oryzae*) is widely used in food industries, and has been evaluated as safe. The media for culturing *A. oryzae* was inexpensive and special equipment was not needed, compared with the culture of insect cells. At the same time, there are many examples of its use in the production of recombinant proteins where high productivity has been achieved.^{7–12} We attempted to produce large amounts of reDer fI E(-1)K by *A. oryzae*.

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Abbreviations: A, absorbance (1 cm); *A. oryzae*, *Aspergillus oryzae*; Endo Hf, Endoglycosidase Hf; kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; re-, recombinant; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid

Materials and Methods

Strains, plasmids, and media. *Escherichia coli* strain MV 1190 was used as a host strain for the construction and amplification of plasmids. A nitrate-assimilation-defective mutant, *A. oryzae* niaD 300, was used as a transformation host throughout this study. Plasmid pUNG1 contains the *A. oryzae* niaD gene, the *glaA* promoter,¹³ the *amyB* terminator, and the *amp* gene. *A. oryzae* niaD 300 and plasmid pUNG1 were kind gifts from Professor K. Kitamoto, The University of Tokyo, Japan. DPY medium consisted of 2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH₂PO₄, 0.05% MgSO₄. Minimal medium (pH 5.5) consisted of 0.2% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, 0.001% FeSO₄, 2% glucose.

Plasmid construction. pUC118-Der fI E(-1)K DNA⁶ was digested with *Bam*HI and the DNA fragment containing Der fI E(-1)K DNA was blunted with a blunting kit (Takara Shuzo). The linker sequence of pUNG1 placed between the *glaA* promoter and the *amyB* terminator was digested with *Sma*I. The blunted Der fI E(-1)K DNA and the *Sma*I-digested pUNG1 were ligated with a ligation kit (Takara Shuzo). pUNG1-Der fI E(-1)K in which Der fI E(-1)K DNA was placed downstream of the *glaA* promoter was used for transformation of *A. oryzae*.

Transformation. Transformation of *A. oryzae* was done as described by Unkles *et al.*¹⁴ To obtain homokaryons of *A. oryzae* transformants, three cycles of sporulation were done on minimal medium.

Southern blot analysis. Transformants of *A. oryzae* were grown at 30°C for 2 days in 10 ml of minimal medium. The mycelia were frozen with liquid nitrogen, lyophilized, and crushed finely by the tips of a pincette. Whole DNA was extracted with 1.5 ml of extraction buffer (37 mM Tris-HCl (pH 8.0), 14 mM EDTA, 3% (w/v) SDS) and purified by phenol extraction, ethanol precipitation, and RNase A treatment. The purified whole DNA was digested with *Xba*I. The digested DNAs were electrophoresed in 1.0% agarose gel and transferred to a nylon membrane (Hybond-N; Amersham). Southern blot analysis was done by Amersham's instructions. The 1.1-kb *Bam*HI fragment of pUC118-Der fI E(-1)K was used as a probe for the detection of Der fI E(-1)K DNA. An improved chemiluminescence system (Amersham) was used for detection according to the manufacturer's instructions.

Production of reDer fI E(-1)K in liquid culture. Approximately 10⁵ conidia of the transformants were inoculated into 10 ml of DPY media and grown at 30°C for 2 days. Transcription from the *glaA* promoter was induced by dextrin in DPY media.¹⁵

Purification of processed reDer fI E(-1)K from liquid culture of the transformant. The culture medium was centrifuged at 1500 × *g* at 4°C for 10 min. The super-

natant was treated with Endo Hf (Biolabs), which was a recombinant fusion protein of Endoglycosidase H and maltose binding protein, at a final concentration of 10 units/ml for 3 h at 37°C. The sample was put directly on a strong anion-exchange column, QMA (Waters), equilibrated with 20 mM phosphate buffer (pH 6.0). After addition of lysylendopeptidase at a final concentration of 10 µg/ml to the flow-through fraction, it was dialyzed against 50 mM Tris-HCl (pH 9.0) overnight at 4°C, and applied directly to a DEAE-Sephacel column (Pharmacia) equilibrated with 50 mM Tris-HCl (pH 8.0). The column was washed with the same buffer, and the adsorbed proteins were eluted by 0–200 mM NaCl concentration gradient. The processed reDer fI E(-1)K was eluted mainly with 50 mM Tris-HCl (pH 8.0) containing 120 mM NaCl. The fractions containing reDer fI were analyzed by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) and by Western blotting. Highly purified fractions were collected.

Production of reDer fI E(-1)K in wheat bran culture. Wheat bran medium was prepared by mixing wheat bran and 0.1 M ammonium sulfate in the ratio of 1:0.8 (w/v), and autoclaved (121°C, 15 min). The medium was inoculated with the conidia (10⁶ conidia/g) of the transformant and incubated at 30°C for 2 days.

Purification of processed reDer fI E(-1)K from the wheat bran culture of the transformant. After the addition of distilled water (1:1 (v/v)), the culture was incubated for 1 h at 4°C. The cultures were filtered with a glass microfiber filter (Whatman) and a 0.45 µm-pore membrane filter (Advantec) successively. Then 1/30 volume of 1.5 M citric buffer (pH 5.5) was added to the filtrate, and Endo Hf-treatment was done at a final concentration of 10 units/ml for 3 h at 37°C. Ammonium sulfate was added to obtain a 60% saturated solution and it was incubated overnight at 4°C. The precipitant formed was collected by centrifugation at 15000 × *g* for 10 min at 4°C, dissolved in 20 mM phosphate buffer (pH 6.0), and passed through a DEAE-Sephacel column equilibrated with the same buffer. After addition of lysylendopeptidase into the flow-through fraction at a final concentration of 10 µg/ml, it was dialyzed against 50 mM Tris-HCl (pH 9.0) overnight at 4°C, and put on a DEAE column equilibrated with 50 mM Tris HCl (pH 8.0). The column was washed with the same buffer, and the adsorbed proteins were eluted with a NaCl concentration gradient. The fractions containing reDer fI were analyzed by SDS-PAGE and by Western blotting. Highly purified fractions were collected.

Trimming of the sugar-chains on reDer fI E(-1)K by Endo Hf. To trim the sugar-chains under reducing conditions, 1/10 volume of denaturing buffer (5% SDS, 10% 2-mercaptoethanol) was added to the sample, and it was boiled for 10 min. Endo Hf-treatment was done at the final concentration of 1 unit/ml for 1 h at 37°C (Fig. 1, panel B and Fig. 2). To trim the sugar-chains under non-reducing conditions, 1/30 volume of 1.5 M citrate buffer (pH 5.5) was added to the sample, and Endo

Hf-treatment was done at the final concentration of 10 units/ml for 3 h at 37°C (Fig. 3, panel A and C).

Concentration of proteins by the trichloroacetic acid (TCA) method. One-tenth volume of 98% TCA was added to the supernatants and they were placed on ice for 10 min. Pellets were collected after centrifugation at $15000 \times g$ for 10 min at 4°C and washed with the same volume of acetone and centrifuged at $15000 \times g$ for 10 min at 4°C. The pellets were dried at room temperature, and dissolved in the appropriate buffer solution.

Skin-prick test. The skin prick test was done by the method of Dredog.¹⁷⁾ Solutions of native Der fl and

processed reDer fl E(-1)Ks from liquid culture and wheat bran culture ($1.0 \mu\text{g}/20 \mu\text{l}$) were applied.

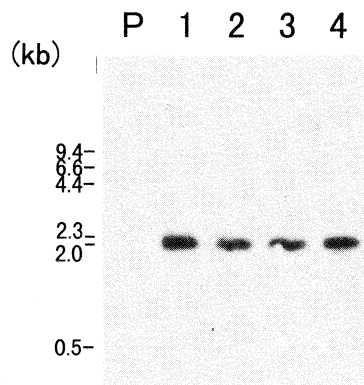
Other methods. SDS-PAGE, Western blot analysis, and assays of IgE-binding activity were done by the methods described previously.⁶⁾

Results

Production of reDer fl E(-1)K in liquid culture

We introduced pUNG1-Der fl E(-1)K into *A. oryzae* and chose four transformants (TF-1, TF-2, TF-3, and TF-4) randomly. When we did genomic Southern blot analysis, no signal was seen in the lane containing DNA of the parental strain (Fig. 1, panel A, lane P) and a signal of 2.0 kb in each lane of transformant DNA was detected (lane 1–4). The intensities of the signals in these lanes were almost the same. Western blot analysis of the culture supernatants of these transformants was done with rabbit anti-Der fl anti-serum. No signal was seen in the culture supernatants of the parental strain (data not shown). Signals were recognized in the supernatants of all the transformants (Fig. 1, panel B, lane 1–4). However, there were the major signal of 18 k in each lane, which is smaller than native Der fl (28 k) and a minor signals of about 35 k corresponding to the size of Der fl E(-1)K DNA. Because it is reported that the addition of NH_4Cl to the media repressed the secretion of proteases from the host,¹⁶⁾ we added NH_4Cl to the medium during cultivation. However, the size of the major signal in the supernatant of cultures of transformants detected by Western blot analysis was 18 k (data not shown). Because the amounts of reDer fl E(-1)K produced by these transformants were almost the same (Fig. 1, panel B, lane 1–4), we chose TF-1 for the production of reDer fl E(-1)K.

A



B

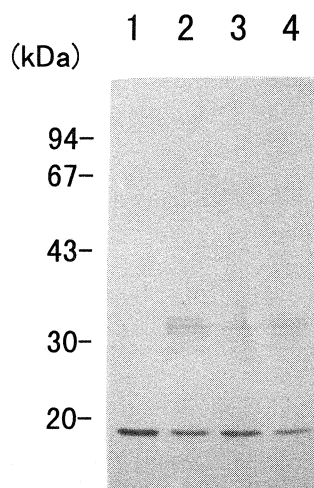


Fig. 1. Characterization of the Transformants.

A. Whole DNA ($2 \mu\text{g}$) was digested with *Xba*I and electrophoresed in 1.0% agarose gel. Genomic Southern blot analysis was done with a probe for Der fl E(-1)K DNA (see Materials and Methods). Lanes: P, the parental strain; 1, TF-1; 2, TF-2; 3, TF-3; 4, TF-4. B. Proteins in the culture supernatants ($200 \mu\text{l}$) of these transformants were concentrated with TCA, treated with Endo Hf under reducing condition, and analyzed by the Western blotting. Lanes: 1, TF-1; 2, TF-2; 3, TF-3; 4, TF-4.

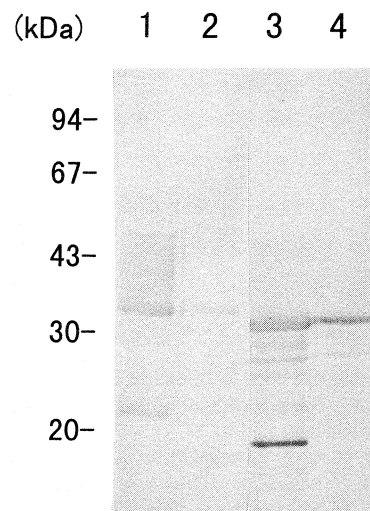


Fig. 2. Production of reDer fl E(-1)K by an *A. oryzae* Transformant (TF-1) under Various Culture Conditions.

Proteins in the culture supernatant (1 ml) were concentrated with TCA, and analyzed by the Western blotting. Lanes: 1 and 2, samples without Endo Hf treatment; 3 and 4, samples treated with Endo Hf under reducing conditions; 1 and 3, samples from a vigorously shaken culture; 2 and 4, samples from a gently shaken culture.

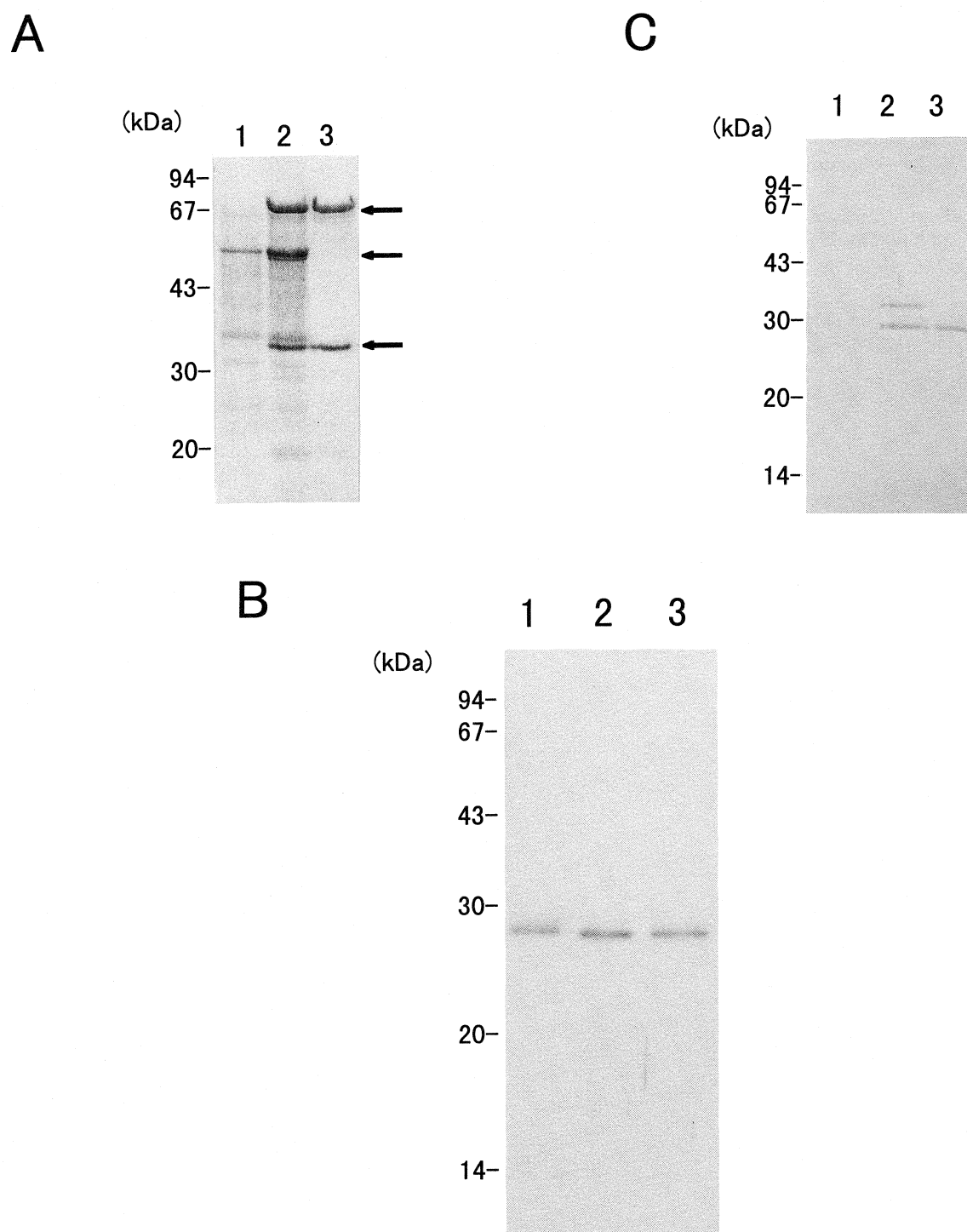


Fig. 3. Monitoring of reDer fI E(-1)K in Purification Processes.

A. Proteins in the supernatant of gently shaken liquid culture of TF-1 were analyzed by SDS-PAGE. Lanes: 1, Proteins in the supernatant (200 μ l) were concentrated by TCA; 2, Proteins in the supernatant (200 μ l) was treated with Endo Hf under non-reducing conditions and concentrated by TCA; 3, Proteins in the supernatant (10 ml) was treated with Endo Hf under non-reducing conditions and passed through a QMA column. The volume of the flow-through fraction became 20 ml and 400 μ l of the flow-through fraction was concentrated by TCA. The upper arrow shows the position of Endo Hf. The middle arrow shows the position of α -amylase. The lower arrow shows the position of reDer fI E(-1)K. B. 0.5 μ g of native Der fI and processed reDer fI E(-1)Ks obtained from cultures of TF-1 were analyzed by SDS-PAGE. Lanes: 1, native Der fI; 2, processed reDer fI E(-1)K obtained from gently shaken liquid culture; 3, processed reDer fI E(-1)K obtained from wheat bran culture. C. reDer fI E(-1)K obtained from wheat bran culture of TF-1 was analyzed by the Western blotting. Lanes: 1, wheat bran culture (5 g) was extracted and the volume of the extract solution became 30 ml. 15 μ l of the extract solution was analyzed; 2, the extract solution of lane 1 (30 ml) was treated with Endo Hf under non-reducing conditions and the volume of Endo Hf-treated solution became 31 ml. 15.5 μ l of the Endo Hf-treated solution was analyzed; 3, Endo Hf-treated solution of lane 2 (31 ml) was treated with lysylendopeptidase and the volume of lysylendopeptidase-treated solution became 40 ml. 20 μ l of lysylendopeptidase-treated solution was analyzed.

To investigate the optimum conditions for the production of reDer fl E(-1)K, we changed the culture conditions. When the culture of TF-1 was shaken vigorously (120 rpm), the transformants grew fast and became spherical shapes. When shaken gently (60 rpm), they grew slower and became threadlike shapes at the bottom of the test tubes. Their culture supernatants were concentrated with TCA and treated with Endo Hf. Although the total amount of reDer fl E(-1)K secreted in the gently shaken cultures was less than that in the vigorously shaken cultures, the signal of the small-size product (18 k) disappeared (Fig. 2, lane 4). Instead, signals corresponding to molecular weight between 35 k and 28 k were recognized. Especially, a signal of 35 k was mainly seen. The change of the size of the signal detected by Western blot analysis due to the change of culture conditions was observed in the case of all four transformants (data not shown). The rate of degradation of reDer fl E(-1)K in lane 3 of Fig. 2 was different from that of lane 1 of panel B of Fig. 1. Since they were not the same samples, we thought that the difference of the rate of degradation was derived from culture condition. When samples without Endo Hf treatment were analyzed (Fig. 2, lane 1 and 2), a major signal of 37 k was recognized, and many signals between 37 k and 50 k were seen. Since no signal was detected in the condensation of the supernatant of liquid culture of parental strain by Western blot analysis, reDer fl E(-1)K produced by *A. oryzae* might be modified with bigger sugar-chains than those of native Der fl.

Purification of processed reDer fl E(-1)K from gently shaken liquid culture of TF-1

Although the degradation of reDer fl E(-1)K was repressed by gently shaken culture, the presence of a large amount of α -amylase in the Endo Hf-treated solution made it difficult to purify reDer fl E(-1)K. When the Endo Hf-treated solution was passed through the QMA column, the reDer fl E(-1)K was not adsorbed to the column but α -amylase was adsorbed (Fig. 3, panel A). By treatment with lysylendopeptidase, the pro-sequence of reDer fl E(-1)K was processed, and most of the other proteins were degraded (data not shown). To avoid the degradation of Endo Hf, Endo Hf treatment was done before lysylendopeptidase treatment. Since these treatments raised the proportion of processed reDer fl E(-1)K in the solution, it was then easily purified with a single anion-exchange column. The extent of the purification was greater than 90% as measured by SDS-PAGE (Fig. 3, panel B, lane 2). The size of processed reDer fl E(-1)K purified from gently shaken liquid culture was a little smaller than that of native Der fl. The overall yield was 0.8 mg from 100 ml of culture supernatant (8 mg/liter).

Purification of processed reDer fl E(-1)K from wheat bran culture

To test the production of reDer fl E(-1)K in the wheat bran culture of TF-1, the extract of the culture was analyzed by Western blotting. No signals were seen (Fig. 3, panel C, lane 1) but signals of two products (35 k, 28 k)

were detected after treatment with Endo Hf, (lane 2) and only one signal was seen after successive treatments with Endo Hf and lysylendopeptidase (lane 3). These data suggest that reDer fl E(-1)K produced in the wheat bran culture is also modified with heterogeneous sugar-chains and the pro-sequence of reDer fl E(-1)K is processed by treatments with lysylendopeptidase. Because a large amount of colored materials, low molecular weight substances, and proteins made it difficult to purify reDer fl E(-1)K, we removed them by ammonium sulfate precipitation, anion-exchange chromatography, and lysylendopeptidase treatment. After these treatments, processed reDer fl E(-1)K was purified with a DEAE-Sephacel column. The purity was greater than 90% as measured by SDS-PAGE (Fig. 3, panel B, lane 3). The processed reDer fl E(-1)K obtained from wheat bran culture was the same size as processed reDer fl E(-1)K isolated from gently shaken liquid culture and was a little smaller than native Der fl. The overall yield was 0.48 mg from 10 g of culture medium (48 mg/kg).

Characterization of processed reDer fl E(-1)K I

The N-terminal amino acid sequence of each processed reDer fl E(-1)K obtained from the supernatant of gently shaken liquid culture of TF-1 and the extract of wheat bran culture of TF-1 was the same as that of native Der fl (Thr-Ser-Ala-Cys-Arg-). The IgE-binding activity of each processed reDer fl E(-1)K obtained from each culture was also the same as that of native Der fl (Fig. 4). The extent of the wheal and flare reaction on the skin elicited by processed reDer fl E(-1)K obtained from each culture was the same as that elicited by native

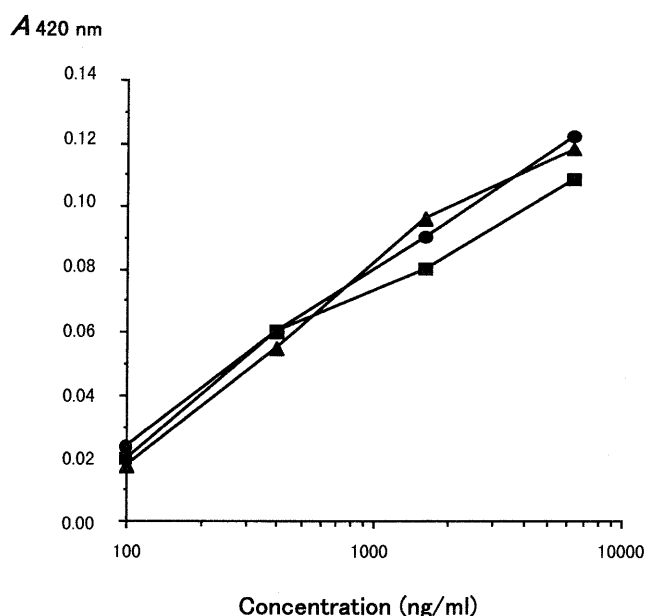


Fig. 4. IgE-binding Activity of Native Der fl and Processed reDer fl E(-1)Ks Obtained from Cultures of TF-1.

The IgE-binding activity of Der fl and processed reDer fl E(-1)Ks is shown. Native Der fl (●); processed reDer fl E(-1)K from gently shaken liquid culture (▲); processed reDer fl E(-1)K from wheat bran culture (■). The relative absorbance was calculated with the value using phosphate-buffered saline (PBS) instead of native Der fl or processed reDer fl E(-1)Ks taken as zero.

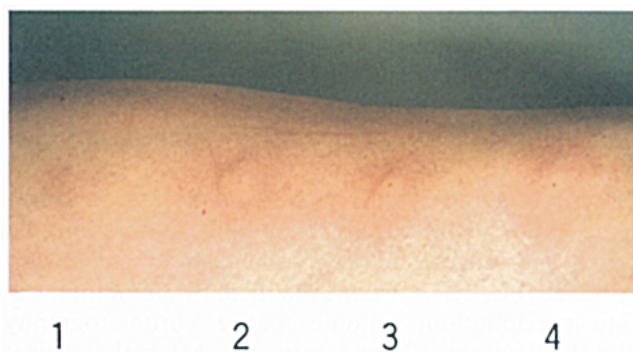


Fig. 5. Flare and Wheal Responses to Native Der fI and Processed reDer fI E(-1)Ks Obtained from Cultures of TF-1 upon Skin-prick Testing.

Wheal responses and flare responses are shown. 1, PBS; 2, native Der fI; 3, processed reDer fI E(-1)K from gently shaken liquid culture; 4, processed reDer fI E(-1)K from wheat bran culture.

Der fI (Fig. 5).

Discussion

When we did genomic Southern blot analysis, a signal of 2.0 kb was detected in each of all four transformants. The signal was thought to be derived from the whole Der fI E(-1)K DNA fragment of pUNG1-Der fI E(-1)K because the 2.0 kb-fragment containing all Der fI E(-1)K DNA sequence was generated when pUNG1-Der fI E(-1)K was digested with *Xba*I. It suggests that intact Der fI E(-1)K DNA is integrated into the genome in each of all four transformants. Since the intensities of signals of all transformants detected by genomic Southern blot and Western blot were almost the same, the copy numbers of Der fI E(-1)K DNA were likely to be the same, and the copy numbers of Der fI E(-1)K DNA were thought to be one by the analysis of genomic Southern blot using *niaD* gene fragment as a probe. The production of reDer fI E(-1)K may be increased if we introduce more copies of Der fI E(-1)K DNA into the host genome using the marker genes which usually integrate at high copy number in parental strain. It seems also important to produce reDer fI E(-1)K as a fused protein^{12,19,20} to obtain higher yields.

In liquid culture, low molecular weight products were detected when cultures of the transformant were shaken vigorously. Since these products reacted to anti-Der fI antibody, these products were probably derived from degradation of reDer fI E(-1)K by the proteases secreted from the host strain. The use of protease-deficient strains as hosts¹⁸) or a medium in which degradation of secreted proteins occurs rarely¹⁹) might be effective for the repression of degradation of reDer fI E(-1)K.

The molecular weight of native Der fI was larger than that of each processed reDer fI E(-1)K obtained from the supernatant of gently shaken liquid culture of TF-1 and the extract of wheat bran culture of TF-1. We thought that the difference of molecular weight was derived from the difference of the size in sugar-chains since native Der fI was not treated with Endo Hf. The sugar-chains of native Der fI were different from that of reDer

fI E(-1)K produced by *A. oryzae*. But the IgE-binding activity and the reaction on the skin of processed reDer fI E(-1)Ks produced by *A. oryzae* were the same as those of native Der fI. This result suggests that both reactions are not affected by the difference in sugar-chains of these products.

Although the yield of reDer fI E(-1)K from gently shaken culture of TF-1 (8 mg/l) was lower than that with a baculovirus expression system (25 mg/l),⁶) the production of reDer fI E(-1)K with *A. oryzae* overwhelmed that with a baculovirus expression system for the reasons described below. To cultivate insect cells, we use special expensive media containing fetal bovine serum. Since the growth rate of the insect cells is slow, we have to spend a long time to get a large amount of insect cells required for mass production. On the other hand, the amplification of an *A. oryzae* transformant could be easily done. The equipments and media for cultivating *A. oryzae* at large scale are very inexpensive compared with those for insect cells.

In this paper we showed the easy way to scaleup for production of processed reDer fI E(-1)K, which was free from the contamination of toxic substance from the host, and which had the same IgE-binding activity or skin reaction as native Der fI. This processed reDer fI E(-1)K will be very useful for many kinds of further experiments, for example, diagnostic applications and immunotherapy.

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