

Production of Recombinant Der fI with the Native IgE-Binding Activity Using a Baculovirus Expression System

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Der fI is a cysteine protease contained in feces of mites and is one of major mite allergens. Recombinant Der fI (reDer fI) that is produced using a baculovirus expression system contains pro-sequences of different lengths. Most of these can be removed by acid treatment. However, IgE-binding activity of acid-treated reDer fI is lower than that of native Der fI at high protein concentrations, and N-terminal amino acids of acid-treated reDer fI are not uniform. Now, a method for processing of the pro-sequence has been developed by producing reDer fI E(–1)K with baculovirus expression system in which the carboxy terminal amino acid of the pro-sequence (glutamate) was replaced by lysine using site directed mutagenesis. No difference in the amount of production was observed upon introducing the mutation into the pro-sequence. Addition of lysylendopeptidase into the culture medium led to processing of the pro-sequence of reDer fI E(–1)K and proceeded the degradation of the other proteins in the medium. Lysylendopeptidase-treated reDer fI E(–1)K was easily purified with an anion exchange column, resulting in 20% increase of the yield. Lysylendopeptidase-treated reDer fI E(–1)K obtained through these processes was compared with the native Der fI. Although some differences were found in protease activity and reactivity with lectins, their N-terminal amino acid and the IgE-binding activity were the same as those of the native one, indicating its usefulness for diagnostic purpose.

Key words: allergen; baculovirus expression system; lysylendopeptidase; lectin

Patients suffering from allergy increase year by year currently. A lot of things that induce allergy by being taken into our bodies from outside are called allergens, pollen and house dust for example. *Dermatophagoides farinae* is a mite in house dust and produces a major proteinaceous allergen, Der fI, which has been proved to react with the majority of patients allergic to mites.¹⁾ Der fI is a cysteine protease contained in the feces of the mite and is thought to function as a digestive enzyme in the intestine of mites.²⁾

Even if the physiological role of Der fI is going to be studied, there is a serious limitation in obtaining large amounts of Der fI because the growth rate of mites is slow and the purification procedure of native Der fI is complex.³⁾ cDNA coding for Der fI has been isolated,⁴⁾ and from the sequence of Der fI cDNA it is proposed that Der fI is produced as a precursor form of a pre-sequence of 18 amino acids, a pro-sequence of 80 amino acids, and a mature-sequence of 223 amino acids.⁴⁾ Native Der fI that is purified from mite culture is a mature protein. Production of reDer fI was tried with a baculovirus expression system.⁵⁾ reDer fI was secreted into culture media when insect cells were infected with recombinant baculovirus in which Der fI cDNA was placed under the polyhedrin promoter. reDer fI was comprised of two types of pro-proteins, each of which includes a pro-sequence of different length: one has a pro-sequence of 80 amino acids and the other has a pro-sequence of 59 amino acids. reDer fI had only 20% of the

IgE-binding activity of native Der fI. Most of their pro-sequences were processed under acidic conditions (pH 4.0, 4°C, 2 days). However, in the removal of the pro-sequence by this method, N-terminal amino acids did not become uniform.⁵⁾ Acid-treated reDer fI was comprised of two types of mature-type proteins: one starting at Ala-[–2] and the other starting at Thr-[1], in which [1] indicates the N-terminal amino acid of native Der fI. IgE-binding activity of acid-treated reDer fI had the same IgE-binding activity as that of native Der fI at low protein concentrations (100 ng/ml) but it was lower than that of native Der fI at high protein concentrations (400 ng/ml).⁵⁾

In this research, we produced reDer fI E(–1)K, using a baculovirus expression system, to let the N-terminal amino acid sequence of mature-type reDer fI become uniform and the same as that of native Der fI. Then we processed the pro-sequence of reDer fI E(–1)K and digested other proteins by lysylendopeptidase during purification. Lysylendopeptidase-treated reDer fI E(–1)K was easily purified and had the same IgE-binding activity as that of native Der fI, and the yield was increased.

Materials and Methods

Cell culture. *Spodoptera frugiperda* Sf9 cells (Invitrogen) were cultured at 27°C in Sf900II medium (Gibco-BRL) with 1% (v/v) fetal bovine serum (FBS). Cells were routinely passed every 3 days.

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Abbreviations: A, absorbance (1 cm); ConA, Concanavalin A; FBS, fetal bovine serum; kDa, kilodalton; LCA, *Lens culinaris* agglutinin; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline; RCA120, *Ricinus communis* agglutinin; re-, recombinant; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin.

Site-directed mutagenesis. The polymerase chain reaction (PCR) technique was used to amplify a DNA fragment coding for Der fI E(–1)K, in which the glutamate codon (GAA) at the C-terminus of the pro-sequence was converted into a lysine codon (AAA) (Fig. 1, panel B). Der fI cDNA inserted into the *Bam*HI site of pUC118 was used as a template. PCR was done to amplify the mutated prepro-sequence using M13 primer M4 as a forward primer and 5'-GCAAGCGCTTGTGTTTGGCATTCAAATCG-AA-3' as a reverse primer (Fig. 1, panel B, underlined). Heat denaturation of the template, annealing, and elongation were done successively for 3 min, 93°C for 2 min, 53°C for 1 min, 72°C, and the reaction was done for 30 cycles. The amplified fragment was digested with *Bam*HI. *Aor*51HI. Der fI cDNA was digested with *Bam*HI, *Aor*51HI to obtain mature-sequence DNA. These fragments were ligated into the *Bam*HI site of pUC118 to make DNA coding for Der fI E(–1)K. The DNA sequence was analyzed with a DNA sequencer WS-10A (Hitachi).

Construction of recombinant virus. Der fI cDNA and the DNA coding for Der fI E(–1)K were ligated into the *Bam*HI site of transfer vector pBlueBacIII (Invitrogen). These transfer vectors and *Autographa californica* nuclear polyhedrosis virus DNA (Invitrogen) were cotransfected into Sf9 cells using Lipofectin (Gibco-BRL) according to the manufacturer's instructions. The blue plaques by coloring with X-gel were picked up because the recombinant virus had *lacZ*. This work was done three times. The production of reDer fI or reDer fI E(–1)K, in the culture medium, was monitored by Western blot analysis.

Production of reDer fIs. Exponentially growing Sf9 cells were infected with recombinant virus at a multiplicity of 5 plaque-forming units/cell. The virus was left to adsorb for 1 h at room temperature with gentle rocking. The inoculum was removed and replaced with Sf900II without FBS and incubated at 27°C for 3 days.

Purification and activation by acid-processing of reDer fIs. Purification and activation by acid-processing of reDer fIs were done by the method of described previously.⁵⁾

Processing of pro-sequence and degradation of other proteins in supernatant of culture media by lysylendopeptidase. The culture medium at 72 h after infection was centrifuged at 1000 × *g* at 4°C for 10 min. The supernatant of the culture was changed to 50 mM Tris-HCl (pH 9.0) by dialysis at 4°C overnight after final 10 µg/ml of lysylendopeptidase (Wako) was added.

Purification of reDer fI E(–1)K after lysylendopeptidase treatment. The solution treated with lysylendopeptidase was put directly on a DEAE-Sephacel (Pharmacia) column that was equilibrated with 50 mM Tris-HCl (pH 8.0). The column was washed with 50 mM Tris-HCl (pH 8.0). The adsorbed proteins were eluted by 0–200 mM NaCl concentration gradient. The fraction containing reDer fI was detected by Western blot. Purity was monitored by SDS-PAGE. Lysylendopeptidase-treated reDer fI E(–1)K was eluted with 50 mM Tris-HCl (pH 8.0) containing 120 mM NaCl.

Measurement of IgE-binding activity. Fifty µl of each reDer fI and native Der fI were coupled to cyanogen bromide activated paper discs. Each disc was washed by 0.1 M NaHCO₃ solution and incubated for 3 h at room temperature in a 1 M 2-ethanolamine solution (pH 9.0). The discs were washed successively by 0.1 M NaHCO₃, 0.1 M acetate (pH 4.0), and PBS containing 0.1% Tween-20. The washed discs were shaken for 3 h at 37°C with human anti-mite IgE serum, washed by PBS containing 0.1% Tween-20, and incubated for 12 h at room temperature with β-galactosidase-conjugated anti-human IgE antibody (Pharmacia). The discs were washed by PBS containing 0.1% Tween-20, and reacted with 0.2 ml of 1.45 mg/ml 2-nitrophenyl-β-D-galactopyranoside (Merck) diluted with 0.05 M phosphate buffer (pH 7.4) containing 0.05% Tween-20. Two ml of 0.1 M NaHCO₃ was added to the reaction mixture, and absorbance (*A*) at 420 nm was measured.

Measurement of cysteine protease activity. Cysteine protease activity was measured by the method of Ando *et al.*⁶⁾ In short, Boc-Val-Leu-Lys-MCA (Peptide institute) was diluted to a final 10 mM with dimethyl sulfoxide and diluted 80 times with 0.1 M phosphoric acid buffer (pH 6.0). This solution was used for substrate. 0.8 ml of substrate, 0.1 ml of 10 mM cysteine solution, 0.1 ml of sample were mixed and incubated at 37°C for 30 min. The reaction was stopped by addition of 2 ml of 15% acetic acid solution.

The fluorescence of aminomethylcoumarin released from the substrate was measured at excitation, 380 nm, and emission, 460 nm, on a fluorometer. The fluorescence degree of 10^{–6} M AMC (Peptide institute) was taken as 100%.

Measurement of reaction to lectins. Native Der fI and lysylendopeptidase-treated reDer fI were electrophoresed and transferred to PVDF membranes. The blocking reaction was done with blocking buffer (10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.05% Tween 20). When wheat germ agglutinin (WGA) was used as a lectin, another blocking buffer (10 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 0.05% Tween 20) was used. The membrane was reacted with 5 µg of biotin-labeled lectin (Honen) which was dissolved in blocking buffer and shaken at room temperature for 1 h. After it was washed with blocking buffer, the membrane was shaken with peroxidase-conjugated avidin solution (Boehringer Mannheim) at room temperature for 15 min. After it was washed with blocking buffer, the membrane was developed with Konica immunostain-HRP (Konica).

Other methods. Analysis of SDS-PAGE, Western blot, and N-terminal amino acid were done as described previously.⁵⁾

Results

Production of reDer fI E(–1)K

First, we intended to measure IgE-binding activity of polypeptides derived from reDer fI treated with various proteases. The IgE-binding activity of reDer fI after lysylendopeptidase at 4°C for 1 day was higher than that of reDer fI but was lower than that of acid-treated reDer fI (data not shown). The molecular weight of reDer fI after lysylendopeptidase treatment at 4°C for 1 day or at 37°C for 1 week became smaller but was larger than that after acid treatment (data not shown). And the molecular weight of acid-treated reDer fI was not changed by lysylendopeptidase treatment at 4°C for 1 day (data not shown). Thus, it was indicated that lysine residues in the mature sequence were not recognized but those in the pro-sequence were recognized by the enzyme (Fig. 1, panel A). Taking advantage of this indication, we replaced the C-terminal amino acid of the prepro-sequence (glutamate) by a lysine residue. For this purpose, PCR was used to amplify a DNA fragment encoding Der fI E(–1)K, in which glutamate codon (GAA) at the C-terminus of the pro-sequence was converted to the lysine codon (AAA) (Fig. 1, panel B). This DNA fragment was placed under the polyhedrin promoter in the transfer vector pBlueBacIII. Recombinant virus was made by cotransfection of the transfer vector and baculovirus DNA into Sf9 cells, and screened by plaque formation. Production of reDer fI E(–1)K or the native reDer fI produced as described previously in the culture medium of logarithmic growing Sf9 cells was measured by Western blot analysis using anti-Der fI rabbit anti serum (Fig. 2). reDer fI and reDer fI E(–1)K were produced in almost the same amount (Fig. 2, lanes 1 and 2). No signal was detected by Western blot analysis in the culture supernatants of Sf9 cells infected with wild type baculovirus or of mock-treated cells (Fig. 2, lanes 3 and 4).

Degradation of proteins in supernatant of culture medium with lysylendopeptidase

Though decomposition products were evident in the culture medium after 3 days (Fig. 2, lanes 1 and 2), we cultured for 3 days after infection to increase the yield. After the supernatant of the culture medium was treated with lysylendopeptidase, its electrophoretic pattern was drastically changed. Almost all proteins were degraded and only

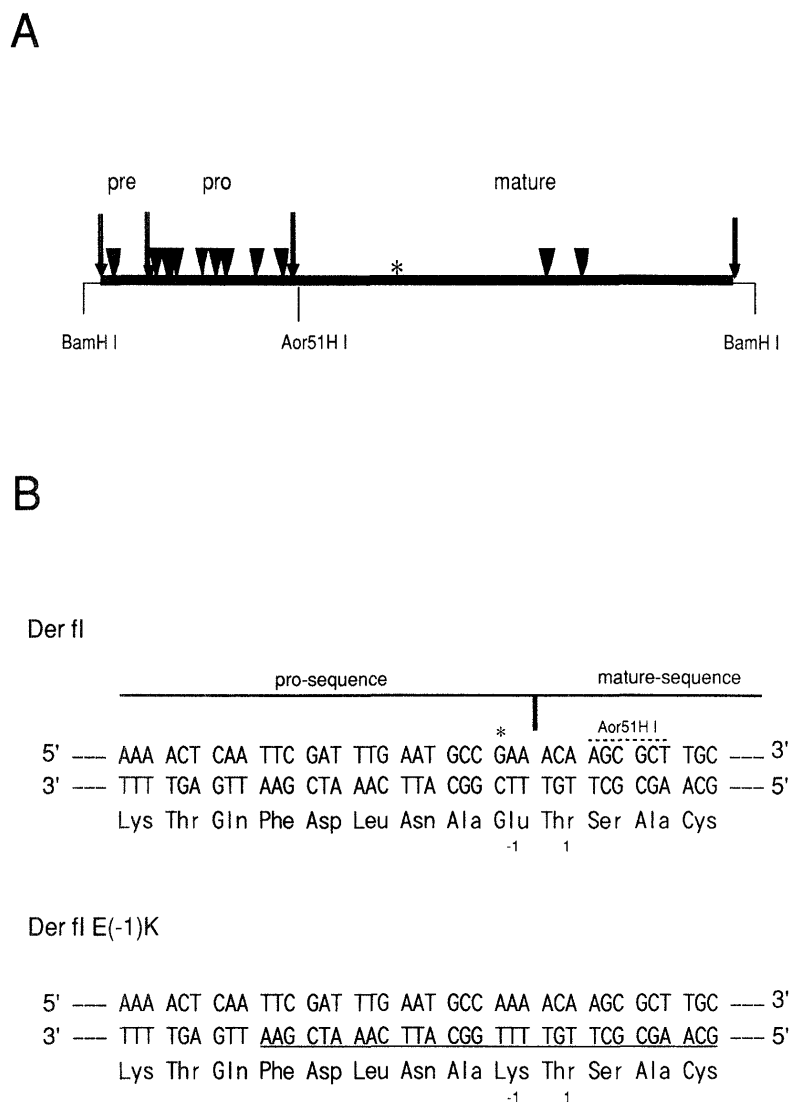


Fig. 1. Positions of Lysine Codons in Der fI and the Strategy for Site-directed Mutagenesis.

A. The thick line shows Der fI cDNA. Arrows show the boundary zone of each sequence. Arrowheads show positions of lysine codons. Asterisk shows the position of *N*-glycosylation site. The recognition sites of restriction enzymes, *Aor51H I* and *BamH I*, are shown by vertical lines. B. Replacement of the C-terminal amino-acid (glutamate codon (GAA)) of pro-sequence by a lysine codon (AAA). Site-directed mutagenesis was done with a PCR method (see Material and Method). Wild type is described as Der fI and mutated type is as Der fI E(-1)K. The nucleotide G that is changed to A is indicated by an asterisk. The recognition site of the restriction enzyme, *Aor51H I*, is shown by a dotted line.

a few new faint bands were seen (Fig. 3, panel A). reDer fI E(-1)K was processed to 28 kDa-protein and unprocessed reDer fI E(-1)K was not seen (Fig. 3, panel B). The amount of reDer fI E(-1)K was not decreased extremely by treatment with lysylendopeptidase and it became the major protein due to severe degradation of the other proteins.

Purification of lysylendopeptidase-treated reDer fI E(-1)K

In our previous research, acid-treated reDer fI was purified through three steps, a DEAE column for purification of the precursor, gel filtration for processing of pro-sequence by acid treatment, and gel filtration for removal of the processed pro-sequence, while lysylendopeptidase-treated reDer fI E(-1)K could be purified through one step, directly putting the lysylendopeptidase-treated supernatant of the culture medium to a DEAE column for purification. In spite of the decrease of purification steps, the purity of lysylendopeptidase-treated reDer fI E(-1)K was almost the same as that of acid-treated reDer fI (Fig. 4). Though the amount of production of reDer fI E(-1)K

was equal to that of reDer fI (Fig. 2), the decrease of the amount of the other proteins by the use of low serum culture, the degradation of most of proteins in the supernatant of the culture medium except for reDer fI E(-1)K by lysylendopeptidase treatment, and the simplicity of purification steps, contributed to increase the yield. As a result, overall yield was 25 mg from 1 liter of the culture medium, which was a 20% increase of that in the previous method.⁵⁾

Characterization of reDer fI E(-1)K

Since acid-treated reDer fI was not processed by lysylendopeptidase and the molecular weight of lysylendopeptidase-treated reDer fI E(-1)K was almost the same as that of acid-treated reDer fI (Fig. 4), it was expected that the pro-sequence of reDer fI E(-1)K was removed by lysylendopeptidase. The N-terminal amino acid of lysylendopeptidase-treated reDer fI E(-1)K was uniform, and it was the same as that of native Der fI (Thr Ser Ala Cys Arg -). Thus, lysylendopeptidase recognized the newly introduced lysine residue at the C-terminus of the pro-

sequence, and the decrease of molecular weight of reDer fI E(-1)K upon addition of lysylendopeptidase showed that the processing of the pro-sequence was done at the same site as native Der fI. Figure 5 shows a comparison of the IgE-binding activity before and after processing of the pro-sequence by either acid treatment or lysylendopeptidase treatment. The IgE-binding activity of both reDer fI and reDer fI E(-1)K were very low. The IgE-binding activity

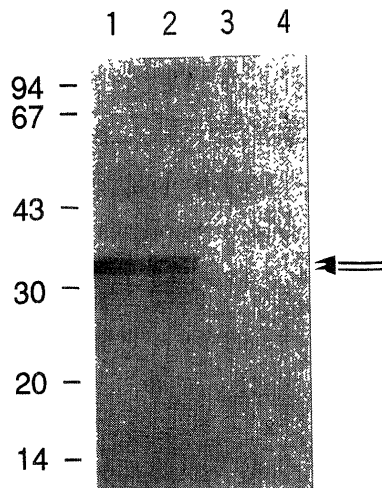


Fig. 2. Production of reDer fI and reDer fI E(-1)K in Culture Media.

Ten ml of the culture medium at 72 h after infection were analyzed by Western blot analysis. Lanes: 1, culture medium of Sf9 cells infected with recombinant baculovirus harboring a DNA fragment encoding Der fI under the polyhedrin promoter; 2, culture medium of Sf9 cells infected with recombinant baculovirus harboring DNA fragment encoding Der fI E(-1)K under the polyhedrin promoter; 3, culture medium of Sf9 cells infected with wild-type baculovirus; 4, mock treated culture. Arrows show 33 kDa and 35 kDa. The numbers on the left mark the positions of the reference proteins (kDa).

of lysylendopeptidase-treated reDer fI E(-1)K was the same as that of the native Der fI. Acid-treated reDer fI had lower IgE-binding activity than the native Der fI and lysylendopeptidase-treated reDer fI E(-1)K at the concentrations of 400 ng/ml and 100 ng/ml. The reason why the IgE-binding activity of acid-treated reDer fI was higher than those of native Der fI and lysylendopeptidase-treated reDer fI E(-1)K at the concentrations of 25 ng/ml was unknown. Before lysylendopeptidase treatment, reDer fI E(-1)K had very low protease activity but lysylendopeptidase-treated reDer fI E(-1)K had protease activity, which was higher than that of native Der fI (Fig. 6). We compared the reactivity of native Der fI and lysylendopeptidase-treated reDer fI E(-1)K to some lectins. Both forms reacted with concanavalin A (ConA) (Fig. 7) but did not react with *Ricinus communis* agglutinin (RCA120) (data not shown), which suggested that both forms were modified by high mannose type sugar chains. Native Der fI reacted with WGA but lysylendopeptidase-treated reDer fI E(-1)K did not (Fig. 7), suggesting that GlcNAc was contained at the non-reducing terminus of the sugar chains of native Der fI but not of lysylendopeptidase-treated reDer fI E(-1)K. Lysylendopeptidase-treated reDer fI E(-1)K reacted with *Lens culinaris* agglutinin (LCA) but native Der fI did not (Fig. 7), suggesting that fucose was connected with GlcNAc, bound to an asparagine residue, but not of native Der fI.

Discussion

IgE-Binding activity of reDer fI treated with lysylendopeptidase, which might contain a pro-sequence of 8 amino acids (Fig. 1, panel B), was lower than that of acid-treated reDer fI (data not shown). It was suggested that short

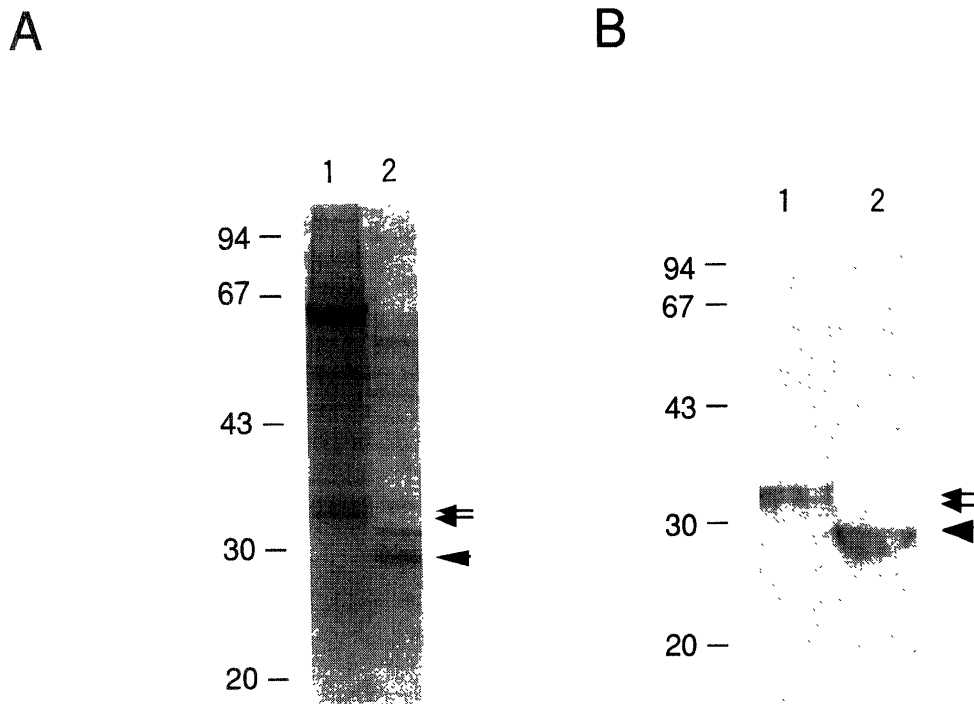


Fig. 3. Degradation of Proteins in Supernatant of Culture Medium with Lysylendopeptidase.

Supernatant of culture medium was treated with lysylendopeptidase. A. The effects of lysylendopeptidase treatment on proteins in supernatant were analyzed by SDS-PAGE. Lanes: 1, culture medium of Sf9 cells infected by recombinant baculovirus harboring DNA fragment encoding Der fI E(-1)K under the polyhedrin promoter; 2, lysylendopeptidase-treated culture medium of lane 1. B. The shift of molecular weight of reDer fI E(-1)K was detected by Western blot analysis. Lanes: 1, culture medium of Sf9 cells infected with recombinant baculovirus harboring DNA fragment encoding Der fI E(-1)K under the polyhedrin promoter; 2, lysylendopeptidase-treated culture medium of lane 1. The two arrows show 33-kDa and 35-kDa proteins for the non-processed reDer fI E(-1)K, and an arrow head shows 28-kDa processed reDer fI E(-1)K. The numbers on the left mark the positions of the reference proteins (kDa).

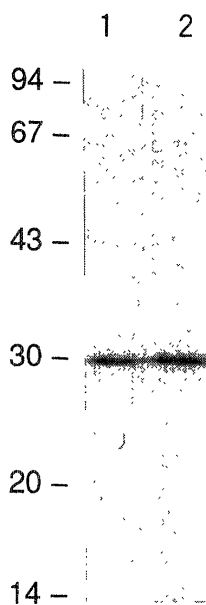


Fig. 4. Comparison of Lysylendopeptidase-treated reDer fI E(-1)K and Acid-treated reDer fI.

The molecular weight of purified lysylendopeptidase-treated reDer fI E(-1)K was compared with that of purified acid-treated reDer fI on SDS PAGE. Lanes: 1, purified lysylendopeptidase-treated Der fI E(-1)K; 2, purified acid-treated reDer fI. The numbers on the left mark the positions of the reference proteins (kDa).

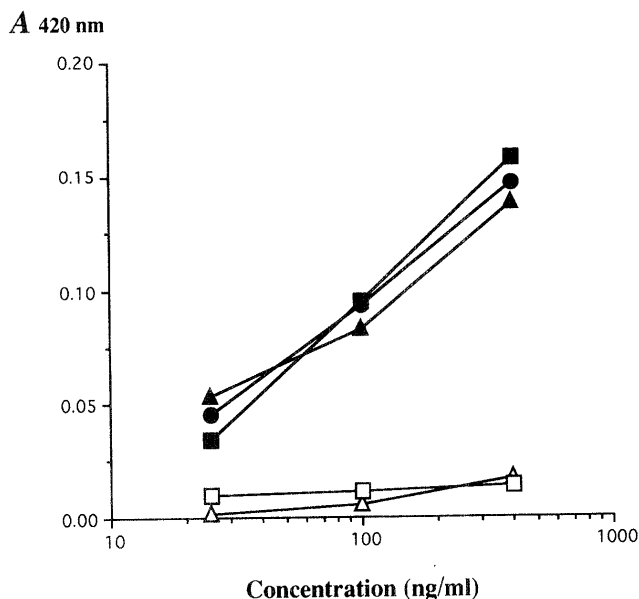


Fig. 5. IgE-Binding Activity of Each Der fI.

IgE-Binding activity of each Der fI is shown. Native Der fI (●); reDer fI without (△) and with (▲) acid treatment; reDer fI E(-1)K without (□) and with (■) lysylendopeptidase treatment

pro-sequence of reDer fI treated with lysylendopeptidase decreased IgE-binding activity. Thus, low IgE-binding activity of acid-treated reDer fI might be derived from 2 amino acids of the pro-sequence presented at the front of the mature sequence in acid-treated reDer fI.

Sometimes proteases were used to trim N-terminal amino acids of recombinant proteins.⁷⁾ Lysylendopeptidase can split many kinds of proteins at the carboxyl side of lysine residues.⁸⁾ However, acid-treated reDer fI was not processed by lysylendopeptidase. Because two lysine residues of reDer fI in mature sequence were contained in a hydrophobic region, we supposed that they were held inside and not recognized by lysylendopeptidase. As a result, lysylendo-

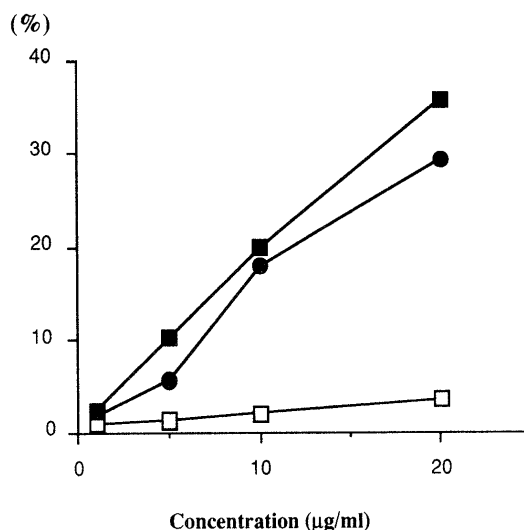


Fig. 6. Protease Activity of Each Der fI.

Protease activity of each Der fI is shown. Native Der fI (●); reDer fI E(-1)K without (□) and with (■) lysylendopeptidase treatment.

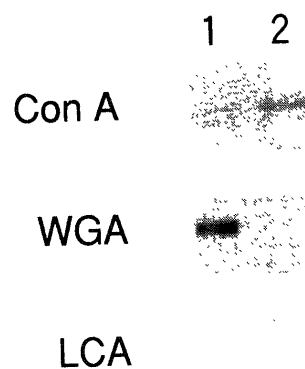


Fig. 7. Reactivity of Native Der fI and Lysylendopeptidase-treated reDer fI E(-1)K with Lectins.

Reactivities of sugar chains of native Der fI and lysylendopeptidase-treated reDer fI E(-1)K with lectins were analyzed (see Material and Method). Lanes: 1, native Der fI; 2, lysylendopeptidase-treated reDer fI E(-1)K. The names on the left mark each lectin.

peptidase could remove the pro-sequence of reDer fI E(-1)K and degrade almost all proteins in the supernatant of the culture media except for reDer fI E(-1)K. The treatment with lysylendopeptidase greatly contributed to the simplification of purification and to the improvement with high grade and high yield. The N-terminal amino acid of the lysylendopeptidase-treated reDer fI E(-1)K was the same as that of the native Der fI. And IgE-binding activity of lysylendopeptidase-treated reDer fI E(-1)K was the same as that of the native Der fI. In this point, lysylendopeptidase treatment is more advantageous than acid treatment.

Though, at the present, whether all sugar chains of reDer fI E(-1)K are different from those of native Der fI is unknown; there is a possibility that the difference of sugar chains would contribute to the difference of protease activities. However, it is also conceivable that the difference of protease activities might be caused by inactivation of a portion of native Der fI at the purification process.³⁾

In this research we have established a simple purification process for reDer fI E(-1)K with high yield, which was about 25 mg from 1 liter of culture broth. A large amount

of production of reDer fI E(-1)K, which would be very useful for diagnostic purposes, immunotherapy, and investigation of participation to allergic development of Der fI, will become possible by this procedure.

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