Cockroach allergen is a major risk factor for IgE-mediated allergic response and asthma in sensitized individuals. Serine proteases have been identified from various sources and characterized as major allergens. The present study was aimed to express and characterize recombinant allergen Per a 10 (rPer a 10) from *Periplaneta americana*. rPer a 10 was expressed in *Escherichia coli* and purified in soluble form, yielding 0.75 mg/liter of culture. Homology of the Per a 10 protein sequence exhibited 27 to 38% similarity to the same serine protease and 41 to 52% similarity to other insect trypsins. The purified rPer a 10 protein resolved at 28 kDa on SDS-PAGE and was recognized by cockroach-hypersensitive patients’ sera by immunoblotting and enzyme-linked immunosorbent assay (ELISA). In competitive ELISA, rPer a 10 required 96 ng of purified protein for 50% inhibition of IgE binding, whereas 34 ng of native protein (nPer a 10) was required for the same inhibition. rPer a 10 and nPer a 10 induced basophil histamine release in the range of 47 to 64% and 60 to 85%, respectively, when sensitized with cockroach-hypersensitive patients’ sera. In conclusion, Per a 10 was subcloned, and the protein was purified to homogeneity. rPer a 10 showed reduced IgE binding and histamine release and showed no proteolytic activity. These data suggest that rPer a 10 has potential for immunotherapy.

**Characterization of Recombinant Per a 10 from *Periplaneta americana***

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Cockroach allergens play an important role in affecting human health through various ways that lead to allergic sensitization causing asthma and rhinitis. *Periplaneta americana* is a common cockroach species that has spread all over the world (1). Immunoblotting of *P. americana* extract showed 22 IgE binding proteins: of those, 9 major IgE binding proteins were identified in individual patient’s sera (2). Only a few allergens, namely Per a 1, Per a 3, Per a 6, Per a 7, Per a 9, and Per a 10, have been purified and characterized from *P. americana* (WHO/IUIS Allergen Nomenclature Sub-Committee, 2012; www.allergen.org). Cockroach extracts are rich in proteases and induce proinflammatory cytokines by airway epithelium (3). Serine proteases were identified as major constituents in guts of *P. americana* (4). Proteases from *Curvularia lunata*, *Epicoccum purpurascens*, *Aspergillus*, and *Penicillium* are important inhalant allergens and have serine protease activity (5, 6). These are also present in house dust mite allergens, such as *Dermatophagoides* allergens Der p 3, Der f 3, and Der p 9 (7).

The proteolytic activity of American cockroach extract was largely due to the presence of serine proteases (8), and it is capable of activating PAR-2 in keratinocytes (9, 10), leading to progression of airway inflammatory diseases, including allergy and bronchial asthma. Per a 10 is a major serine protease allergen from the American cockroach and has shown proteolytic activity and caused inflammation in lungs of mice (11). Per a 10 also modulates the dendritic cell response toward Th2 by upregulating CD86, interleukin-6 (IL-6), and reduced IL-12 secretions (12).

Several cockroach allergens, like trypsin-encoding cDNA (bgtryp-1) from *Blatella germanica* (13) and Per a 1, 3, and 7 from *P. americana*, have been cloned and characterized. Recently a major allergen from *C. lunata* was cloned, expressed, and characterized as subtilisin-like serine protease (14). Recombinant DNA technology has provided the opportunity to study the specific allergenic proteins, which may be modified to reduce allergenicity for safer treatments (15, 16). Proteolytically inactive Per a 10 regulated the inflammatory parameters in mice (11).

The present study was aimed at expression and purification of the serine protease Per a 10, a major allergen from *P. americana*, and is characterized by a bioinformatics approach. The biological activity of recombinant Per a 10 (rPer a 10) and its relevance as an allergen among cockroach-sensitive patients’ sera were determined by immunobiochemical methods.

**MATERIALS AND METHODS**

**Expression and purification of rPer a 10.** The gene coding for Per a 10 (database accession no. **AY792954**) was amplified by a gene-specific primer and subcloned into the EcolI and Xhol sites of the PET 22b vector, expressed in *Escherichia coli* BL21, and purified by Ni-nitrilotriacetic acid (NTA) agarose, as described previously (12). The purified protein was dialyzed, and purity of the protein was determined by SDS-PAGE (12%) gel) and Coomassie brilliant blue (CBB) staining (0.1%). Furthermore, it was analyzed by Western blotting with polyclonal His tag antibody.

**Purification of nPer a 10.** The cockroach (*P. americana*) allergen extract was prepared 1:50 (wt/vol) in phosphate-buffered saline (PBS [pH 7.4]) with phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO). The native Per a 10 (nPer a 10) protein was purified by affinity chromatography as previously described (17).

**Homology search.** The deduced amino acid sequences of Per a 10 protein were compared by the NCBI BLAST search program (http://blast.ncbi.nlm.nih.gov/blast.cgi), and multiple sequence alignment was performed using ClustalW (http://www.ebi.ac.uk/clustalw/).

**CD analysis of nPer a 10 and rPer a 10.** Circular dichroism (CD) spectra of nPer a 10 and rPer a 10 were collected on a Jasco J-710 spectropolarimeter using a 1-mm path-length quartz cuvette at a protein concentration of 100 μg/ml in 20 mM sodium acetate buffer, pH 5.0, containing 200 mM NaCl. The CD spectra were measured at 25 °C and 6°C using a scan speed of 60 nm/min, a bandwidth of 1.0 nm, and an integration time of 0.1 s.

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Characterization of Recombinant Per a 10

TABLE 1 Intradermal test and ELISA results from cockroach-hypersensitive patients

<table>
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<tr>
<th>Serum no.</th>
<th>Age (yr)/sex</th>
<th>Clinical history</th>
<th>i.d. test grade</th>
<th>Cockroach extract</th>
<th>nPer a 10</th>
<th>rPer a 10</th>
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<td>1</td>
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</table>

Table footnote:
- a M, male; F, female.
- b AR, allergic rhinitis; BA, bronchial asthma.
- c +, equivalent to histamine (positive control); ++, more than histamine (positive control).
- d Histamine diphasphate (100 mg/ml) was used as a positive control. Sera showing an OD of ≥3 times the average value of control sera were considered positive (patients 1 to 16). The tests were also carried out on six nonallergic subjects (C1 to C6).

ELISA. A specific IgE enzyme-linked immunosorbent assay (ELISA) was performed to determine the allergenicity of the rPer a 10 along with nPer a 10 and cockroach extract, as described previously (17). A microtiter plate (Nunc) was coated with purified rPer a 10 protein, nPer a 10, or cockroach extract separately, and sera from healthy subjects were used as control. After incubation, the plates were washed with PBS containing 0.05% Tween 20 (vol/vol) twice followed by PBS twice. The plates were incubated with 1:1,000 (vol/vol) diluted anti-human IgE peroxidase (Sigma Chemicals, Co., St. Louis, MO) at 37°C for 3 h and washed with PBS-T (PBS containing 0.05% Tween 20) twice followed by PBS three times. The color was developed with o-phenylenediamine, and the reaction was stopped by adding 50 μl of 3 N H2SO4, and absorbance was read as the optical density at 492 nm (OD492) in an ELISA reader (Benchmark Plus; Bio-Rad).

IgE immunoblot. The binding ability of rPer a 10 to human IgE antibodies from cockroach-hypersensitive patients’ sera was studied by Western blotting. SDS-PAGE-resolved proteins were transferred electrophoretically onto a nitrocellulose membrane in buffer containing 25 mM Tris, 192 mM glycine, and 25% methanol at a constant 50 V overnight at 4°C. The membranes were cut into strips, blocked with 3% defatted milk in PBS, and incubated overnight with 1:10 (vol/vol)-diluted cockroach-hypersensitive patients’ sera (n = 16) containing 0.5% defatted milk at 4°C; sera from healthy subjects were used as the control. After incubation, the strips were washed with PBS containing 0.05% Tween (PBS-T) followed by PBS, probed with 1:1,000 (vol/vol)-diluted anti-human IgE peroxidase (Sigma Chemicals Co., St. Louis, MO) for 3 h at 37°C, and washed with PBS and PBS-T. The IgE-reactive bands were visualized with diaminobenzidine (DAB) and hydrogen peroxide in acetate buffer. The reaction was stopped by washing the strips with deionized water.

ELISA inhibition. The specific IgE inhibition assay was carried out to determine the potency of nPer a 10 or rPer a 10. The microtiter plate was coated with antigen (1 μg of P. americana extract) overnight at 4°C. Cockroach-hypersensitive pooled patient sera were preincubated at 4°C overnight with different concentrations (0.1 to 10,000 ng) of rPer a 10, nPer a 10, or cockroach extract separately, and sera from healthy subjects were used as control. The plates were blocked with 1% bovine serum albumin (BSA) and then incubated with 100 μl of a preincubated mixture of 1:10 (vol/vol)-diluted cockroach-hypersensitive patients’ sera overnight at 4°C. Inhibition is presented as the percentage decrease in absorbance of the sample with inhibitor to that of sample without inhibitor, and allergenic potency was defined by establishing the concentration of protein required for 50% inhibition.

Immuno blot inhibition. Immuno blot inhibition was performed to establish the specificity of rPer a 10 and cockroach extract with other serine protease allergens. Cockroach-hypersensitive pooled patient sera were incubated with 500 μg of cockroach extract, rPer a 10 for homologous inhibition, and Curvularia extract and 31-kDa purified serine protease from Curvularia. The SDS-PAGE-resolved protein was transferred onto nitrocellulose membrane. The bands were visualized with Ponseau-S to ensure the transfer of the proteins. The membranes were cut into strips and blocked with 3% defatted milk in PBS. After blocking, the strips were incubated with the inhibited pooled patients’ sera. The rest of the procedure was similar to that described for the immunoblotting methods.

Zymography. The enzymatic activity of rPer a 10 and nPer a 10 was analyzed by gelatin zymography. The purified protein was fractionated on 12% polyacrylamide gel containing copolymerized 0.1% gelatin as the substrate. The gels were washed with 2.5% (vol/vol) Triton X-100 for 1 h on an orbital shaker to remove the SDS, as described previously (14). The gels were then rinsed twice with deionized water to remove the Triton X-100 and incubated overnight at 37°C in 100 mM Tris-glycine (pH 7.4), stained with CBB, and destained.

Histamine release assay. The histamine release assay was performed as described previously (19) for both active and heat-inactivated protein.
of rPer a 10 and nPer a 10, with cockroach-hypersensitive patients’ sera. The proteins were inactivated by being heated at 95°C for 15 min. The leukocyte separation solution with 6% dextran in saline containing 0.01 M EDTA and 2% dextrose was mixed with the peripheral blood (1:5 [vol/vol]) drawn from healthy subjects, and the mixture was incubated for 90 min at room temperature to separate the leukocyte-rich layer. The separated layer was drawn off carefully without red blood cell (RBC) contamination, centrifuged at 300 × g at 4°C, and then washed twice in saline. The cell count was performed, and the cell suspension containing 2.5 × 10⁶ cells was taken for incubation. The bound IgE was stripped off from the basophil suspension by incubation in lactic acid buffer for 3.5 min at 37°C. The cells were washed in HEPES buffer containing 0.05%, human serum albumin (HSA) (pH 7.4) and incubated with a sensitization mixture containing patient's sera against cockroach allergen (n = 15) at 37°C for 90 min. Cells sensitized with sera from healthy subjects were used as a negative control. After passive sensitization with cockroach-hypersensitive patients’ sera, the basophils were stimulated with 5 ng of cockroach extract or the nPer a 10 or rPer a 10 in HEPES buffer containing 0.05% HSA and 1 mM CaCl₂ at 37°C for 1 h, and simultaneously the same heat-inactivated proteins (95°C for 15 min) were also incubated in HEPES buffer under the conditions described above. After incubation, the reaction was stopped by the addition of 750 μl of ice-cold 0.9% NaCl (wt/vol). Then the cells were centrifuged at 12,000 × g for 8 min, and the cell-free supernatant containing histamine was taken in a test tube and mixed with the peripheral blood (1:5 [vol/vol]) drawn from healthy subjects, and the mixture was incubated for 90 min at room temperature to separate the leukocyte-rich layer. The separated layer was drawn off carefully without red blood cell (RBC) contamination, centrifuged at 300 × g at 4°C, and then washed twice in saline. The cell count was performed, and the cell suspension containing 2.5 × 10⁶ cells was taken for incubation. The bound IgE was stripped off from the basophil suspension by incubation in lactic acid buffer for 3.5 min at 37°C. The cells were washed in HEPES buffer containing 0.05%, human serum albumin (HSA) (pH 7.4) and incubated with a sensitization mixture containing patient’s sera against cockroach allergen (n = 15) at 37°C for 90 min. Cells sensitized with sera from healthy subjects were used as a negative control. After passive sensitization with cockroach-hypersensitive patients’ sera, the basophils were stimulated with 5 ng of cockroach extract or the nPer a 10 or rPer a 10 in HEPES buffer containing 0.05% HSA and 1 mM CaCl₂ at 37°C for 1 h, and simultaneously the same heat-inactivated proteins (95°C for 15 min) were also incubated in HEPES buffer under the conditions described above. After incubation, the reaction was stopped by the addition of 750 μl of ice-cold 0.9% NaCl (wt/vol). Then the cells were centrifuged at 12,000 × g for 8 min, and the cell-free supernatant containing histamine was taken in a test tube and mixed with 200 μl of 12% perchloric acid. The histamine content was determined by the fluorometric method, using o-phthaldialdehyde as the substrate. Spontaneous histamine release was measured in the supernatant of unstimulated cells. The total histamine content was determined by lysis of a separated aliquot of leukocytes with 3% perchloric acid. The allergen-induced histamine release was calculated as a percentage of the total histamine content after correction for spontaneous release.

**Statistical analyses.** Specific IgE and histamine releases in response to rPer a 10 and nPer a 10 were compared by a paired t test, and the correlation coefficients were determined by linear regression.

**RESULTS**

**Expression and purification of Per a 10.** rPer a 10 was expressed in _E. coli_ and affinity purified. The yield of purified rPer a 10 was 3 mg/liter bacterial culture. The purified protein resolved at 28 kDa in 12% SDS-PAGE was shown to have more than 90% purity (Fig. 1). Furthermore, rPer a 10 was recognized by His tag polyclonal antibody. nPer a 10, purified by affinity chromatography from _P. americana_ extract, yielded a single band at 28 kDa in SDS-PAGE (Fig. 1).

**Homology.** On the basis of the BLASTP homology searches, the deduced amino acid sequences exhibited 41 to 52% similarity to insect trypsins. Per a 10 showed high similarity (71%) to the trypsin of _B. germanica_ (Bla g 1 trypsin) and was 27 to 38% similar to mite serine protease allergens like _Bt1_ 3 (_B. tropicalis_), Der p 3 (_Dermatophagoides pteronyssinus_), Der p 9 (_D. pteronyssinus_), and Der f 3 (_Dermatophagoides farinae_) with the fully conserved catalytic domain GDSGG (Fig. 2).

**CD.** The far-UV CD spectra of purified nPer a 10 and rPer a 10 recorded at room temperature (25°C) showed two minima at 207 and 225 nm and a large maximum at 217 nm (Fig. 3). Calculation of the secondary structure using the program K2D2 resulted in 3.13% and 49.32% α-helix and β-sheets, respectively, for nPer a 10 and 19.92% and 27.52% α-helix and β-sheets, respectively, for rPer a 10 (Fig. 3).

**Specific IgE binding of rPer a 10.** The specific IgE binding of rPer a 10 or nPer a 10 was identified by ELISA and immunoblotting using individual cockroach-hypersensitive patient’s sera (n = 16). For immunoblotting, patients’ sera (n = 16) were probed against rPer a 10, and sera from healthy subjects were used as a negative control. All 16 patients’ sera reacted with rPer a 10 protein, indicating it is a major allergen. Sera from healthy subjects showed no IgE binding in the immunoblot (Fig. 4). Cockroach-hypersensitive patients’ sera showed raised IgE values against cockroach extract compared to nPer a 10 and rPer a 10. The specific IgE absorbance values for cockroach extract ranged from an optical density (OD) of 1.506 to 2.125, that of nPer a 10 ranged from OD of 0.812 to 1.3125, and that of rPer a 10 ranged from OD of 0.492 to 0.755. OD values ≥3 times that of the control sera (OD of 0.092) were taken as the cutoff for an ELISA-positive result (Table 1). IgE binding to rPer a 10 is positively correlated to nPer a 10 (R = 0.71).

**Potency of purified protein.** ELISA inhibition was performed to check the allergenic potency and specificity of IgE binding of rPer a 10 compared to that of nPer a 10. The dose-dependent inhibition of IgE binding to the solid phase of cockroach extract was observed. The pooled cockroach-hypersensitive patients’ sera were incubated with different concentrations (0.1 to 10,000 ng) of cockroach extract, nPer a 10, or rPer a 10 protein. A maximum of 90% inhibition was achieved when 1 μg of cockroach extract was used, nPer a 10 10 showed 85% inhibition, and rPer a 10 protein showed 62% inhibition for solid-phase-bound crude cockroach allergen extract. For 50% inhibition (IC₅₀) of solid-phase-bound crude cockroach allergen extract, 96 ng of rPer a 10 protein was required, compared to 34 ng of nPer a 10 and 26 ng of cockroach extract (self) for the same level of inhibition (Fig. 5a).

Immunoblot inhibition was performed to find the specificity of rPer a 10. Preabsorption of pooled cockroach-hypersensitive patients’ sera with cockroach extract and purified protein completely abolished IgE binding to cockroach extract and rPer a 10 protein. With _Curvularia_ as an inhibitor, no cross-inhibition or cross-reactivity was observed in cockroach extract and rPer a 10 protein (Fig. 5b).

**Proteolytic activity of recombinant protein.** The qualitative analysis of rPer a 10 showed that the protein is not enzymatically active; this may be due to improper folding, or conformational changes in rPer a 10 may lead to an inactive form. nPer a 10 showed activity on the zymogram (Fig. 6).

**Histamine release assay.** The biological activity of nPer a 10 or rPer a 10 was assessed by its ability to release histamine from sensitized basophils. Basophils sensitized with cockroach-hyper-
sensitive patients’ sera (IgE) individually showed histamine release on challenge with cockroach extract, nPer a 10, and rPer a 10. Histamine release by individual patient’s sera ranged from 60 to 85% and 47 to 64% upon challenge with nPer a 10 and rPer a 10, respectively, and showed a positive correlation ($R = 0.88$) (see Fig. S1a in the supplemental material), whereas the cockroach extract showed 38 to 52% histamine release (Fig. 7a).

The heat-inactivated nPer a 10 and rPer a 10 showed reduced histamine release. There were histamine releases of 30 to 39% and

FIG 2 Multiple sequence alignment of Per a 10 protein with different mite proteins. Per a 10 showed 27 to 38% homology with mite trypsins.

FIG 3 Circular dichroism spectra of rPer a 10 and nPer a 10. Protein was taken at a concentration of 0.1 mg/ml in 10 mM phosphate buffer, and spectra were recorded in the far-UV range (190 to 260 nm).

FIG 4 Immunoblot of IgE binding with individual patient’s and control sera. Lane M, molecular mass marker; lanes 1 to 16, IgE binding of rPer a 10 protein with individual patient’s sera; and lanes C1 to C6, rPer a 10 with sera from healthy subjects.
27 to 37% with heat-inactivated nPer a 10 and rPer a 10, respectively, which is positively correlated ($R = 0.97$) (see Fig. S1b in the supplemental material), whereas cockroach extract showed 20 to 29% histamine release. Basophils stimulated with control subjects’ sera showed 6 to 9% histamine release in response to nPer a 10, rPer a 10, or cockroach extract (Fig. 7b).

**DISCUSSION**

Allergenic extracts prepared from natural sources for diagnosis and therapies are a complex mixture of proteins and other molecules which may differ qualitatively in various batches. Extracts of a major allergen may be helpful for obtaining a standardized preparation. Furthermore, recombinant allergens are considered safer and more effective than whole-allergen extracts for component-resolved diagnosis and therapy of allergy-specific disorders (15).

In the present study, Per a 10 allergen was subcloned, expressed, purified, and compared with nPer a 10 by immunobiochemical methods.

Homology studies of Per a 10 showed 71% similarity to *B. germanica* and 27 to 38% similarity to mite serine protease allergens like *B. tropicalis*, *D. pteronyssinus*, and *Dermatophagoides farinae*. Serine proteases from fungi and mites were recognized as major allergens by IgE binding studies. The purified nPer a 10 showed 82% IgE binding on immunoblotting and ELISA to the *P. americana*-hypersensitive patients’ sera (17). In the present study, rPer a 10 showed 2-fold reduction (43%) in specific IgE binding to cockroach-hypersensitive patients’ sera in ELISA and immunoblotting compared to >80% reduction with nPer a 10.

The study by Jeong et al. showed that the cockroach extract of *P. fuliginosa* and *B. germanica* showed 83% and 51% inhibition, respectively (20). In the present study, rPer a 10 showed 2.8 times less inhibition than nPer a 10 for reduction in 50% IgE binding on solid-phase-coated cockroach extract. Cockroach allergens are species specific, and group 1 allergens (tropomyosin) like Bla g 1 and Per a 1, are cross-reactive, which have been sequenced from both *P. americana* and *B. germanica* (21). Furthermore, IgE binding of rPer a 10 was completely inhibited when pooled patients’ sera were preabsorbed with 500 ng of self-protein in immunoblot inhibition, and there was no inhibition in *C. lunata* serine protease, which showed clear bands. Serine proteases are reported from many sources, and most of them are cross-reactive allergens (22–24).

The biological activity of recombinant allergens was determined by basophil histamine release assay (14, 25). In this study, rPer a 10 and nPer 10 induced significant histamine release by 47 to 64% and 60 to 85%, respectively, which was positively correlated. The heat-inactivated proteins of nPer a 10 and rPer a 10
induced histamine release by 30 to 39% and 27 to 35%, respectively, which is correlated positively, and release was comparatively less toward the active proteins. The IgE binding and biopotency of rPer a 10 were low compared with that of nPer a 10. This may be due to the fact that the conformational B-cell epitopes in recombinant protein are in reduced form due to its improper folding (26, 27).

Serine proteases in cockroach extract may induce proinflammatory effects on human bronchial epithelial cells, leading to the progression of airway inflammatory diseases (3). Per a 10 has trypsin-like activity (17), and proteolytic activity enhances the entry of other allergens into the epithelium and plays a role in other allergen presentation (11). The absence of proteolytic activity of rPer a 10 may be due to improper folding of the protein. The proteolytically inactive Per a 10 with a reduced Th2 inflammatory response induced IL-10 in a mouse model (28). E. coli-expressed recombinant proteins get aggregated in insoluble form, whereas rPer a 10 is a soluble protein, and ease of purification may have an advantage for clinical use.

Conclusions. Per a 10 was subcloned, and the protein was purified to homogeneity. rPer a 10 showed reduced IgE binding and histamine release and showed no proteolytic activity. These data suggest that rPer a 10 has potential for immunotherapy.

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