

Isolation and *in vitro* translation of messenger RNA from the American cockroach

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Summary

Total RNA was extracted from the whole body of American cockroach (*Periplaneta americana*) using chaotropic salt guanidine isothiocyanate in the presence of 2-mercaptoethanol (2-ME). Polyadenylated mRNA was isolated by oligothymidylic acid-cellulose chromatography and mRNA was translated using a rabbit reticulocyte lysate system. The translation, as judged by the incorporation of ^{35}S -methionine, was obtained with poly(A)⁺ RNA, where an approximately 9.5-fold increase in label incorporation over control was achieved. Analysis of translation products by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in combination with autoradiography showed that many proteins with apparent molecular weights ranging from 12 to 200 kD were synthesized, and no labelled proteins were found with negative RNA control and poly(A)⁻ RNA. Immunoprecipitation studies performed using polyclonal and monoclonal antibodies revealed that synthesized proteins of MW 90, 78, 72, 49, 45, and 26 kD corresponded with previously identified principal and major allergens of American cockroach from our laboratory. In addition, the allergenicity of the translation mixtures was also confirmed by fluoroallergosorbent test (FAST) inhibition studies with IgE antibodies of human reaginic serum pool.

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Introduction

Cockroaches are recognized as one of the major causes of perennial rhinitis and asthma, and high incidences of cockroach hypersensitivity in atopic and asthmatic populations have been reported [1–10]. Several studies have been devoted to the isolation and characterization of allergens, and American cockroach allergens with molecular weights (MW) ranging from 6 to 120 kD have previously been identified by crossed radioimmuno-electrophoresis, immunoblot analysis and radioallergosorbent test inhibition [11–17]. In our previous studies, the partially purified Cr-PI fraction, containing two prominent proteins of 78 and 72 kD that bound 100% of the atopic sera tested, have been identified as the major allergens of American cockroach [11], and monoclonal antibodies (MoAbs) against these two allergens have been generated [18]. No MoAbs reacted with any components of crude extracts of Oriental (*Blatta orientalis*) and

German (*Blattella germanica*) cockroaches as determined by immunoblotting and enzyme immunoassay.

Recently, the impact of the technology of recombinant DNA on immunochemical structure and properties of allergen is best illustrated by the fact that several allergens have been cloned and sequenced [19]. The availability of major allergens of American cockroach synthesized in *Escherichia coli* may allow approaches to the analysis of the physicochemical structure of these allergens complementary to those afforded by the traditional methods of protein biochemistry. The present study describes the isolation and purification of biologically active mRNA from the whole body of American cockroach and the analysis of the products of *in vitro* translation as a preliminary phase of a cDNA-based approach to the molecular cloning of these allergens.

Materials and methods

Isolation of total RNA and purification of mRNA

Batches of live adult insects were trapped, identified and stored in a -70°C deep freezer. The whole body of

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American cockroach was ground in liquid nitrogen, then immediately suspended in guanidine isothiocyanate (50 mM Tris-HCl, pH 7.5; 4.0 M guanidine isothiocyanate and 25 mM EDTA):2-ME (23:2) solution (GuSCN/ME) and homogenized. Debris was removed by centrifugation (7000 g), ethanol was added, and the suspension was centrifuged at 16 000 g for 3 min at 4°C. Supernatant was precipitated with 1.0 M acetic acid:ethanol (1:30) solution, chilled and centrifuged at 7000 g for 10 min at 4°C. The RNA pellet was resuspended in the ice-cold GuSCN/ME solution and dissolved by homogenization, then centrifuged and precipitated again with ethanol. Guanidine hydrochloride (6.0 M guanidine HCl, pH 7.5; 25 mM EDTA):2-ME (1250:1) solution (GuHCl/ME) was added to the RNA pellet and dissolved by trituration with a sterile pipette. RNA was then precipitated with 1.0 M acetic acid:ethanol (1:10) solution thrice. The precipitated RNA was dissolved in binding buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, 0.3 M NaCl, 0.1% SDS) and fractionated by oligothymidylic acid-cellulose chromatography (GIBCO/BRL, Gaithersburg, MD, USA). Absorbance of RNA preparation was determined at 260 nm, and yield was calculated assuming $A_{1\text{ cm}}/0.1\%$ at 260 nm = 25 [20].

In vitro translation

Cockroach mRNA was translated *in vitro* using a rabbit reticulocyte lysate system (Amersham International, Amersham, U.K.) according to the method recommended by the manufacturer. Briefly, the reaction mixture containing 35 µl of lysate, 1.0 µg of mRNA, 2.0 M potassium acetate solution, 1.0 mM amino acid mixtures without methionine and 25 µCi of ³⁵S-methionine in a total volume of 50 µl was allowed to react at 30°C and stopped after 60 min incubation. Aliquots were applied to GF/C filters (Whatman, Maidstone, U.K.) and incubated on ice for 15 min in 5% trichloroacetic acid (TCA) containing 1 mg/ml methionine, and then boiled in this solution for 15 min. The filters were washed and the precipitated radioactivity was counted by Packard 2200CA TRI-CARB liquid scintillation analyser (Packard, Downers Grove, IL, U.S.A.). The translated products from four different experiments were pooled and stored at -70°C.

Preparation of Cr-PI and production of antibodies

Crude American cockroach extract (CRa-A) was obtained by extracting the whole body of insects with CoCa's solution, and Cr-PI was prepared from the crude extract by Sephadex G-150 SF (Pharmacia, Piscataway, NJ, U.S.A.) gel filtration [11]. Polyclonal anti-Cr-PI was

raised by repeated intradermal injection of rabbits with 200–400 µg of Cr-PI, and A-2 and E-4 MoAbs were generated from fusion experiments between spleen cells of BALB/c mice immunized with Cr-PI and murine plasmacytoma NS-1 cells [18].

Immunoprecipitation (IP) of labelled cockroach proteins

³⁵S-methionine-labelled cockroach proteins were precipitated using anti-Cr-PI antibodies and protein A-sepharose suspension [21]. SDS was added to 20 µl of the translation mixture to yield a final concentration of 4% and heated at 100°C for 4 min. Four volumes of dilution buffer (60 mM Tris-HCl, pH 7.4; 190 mM NaCl, 6 mM EDTA, 1.25% Triton X-100 and 10 units/ml Trasylol) and one volume of distilled water were then added to the mixture. After addition of either 4 µl of sodium sulfate purified [11] polyclonal anti-Cr-PI antibody (4.0 mg/ml), 2 µl of Protein A-agarose affinity purified MoAb A-2 (2 mg/ml) and 2 µl of MoAb E-4 (2 mg/ml) [18], or pre-immunized rabbit sera (5 mg/ml), the mixture was incubated at 4°C for 2 hr with gentle mixing, then at 20°C for 15 min with constant shaking. The mixture was then added with 50 µl of well-swollen 3.0% protein A-sepharose in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4) and incubated at 20°C for 45 min with constant shaking. Immune complexes were centrifuged at 3000 g, then washed twice with 100 µl of NET and suspended in sample buffer for SDS-PAGE analysis.

SDS-PAGE and autoradiography

SDS-PAGE was performed in a 12% gel in conjunction with 4% stacking gel according to the method of Laemmli [22] as described [11]. Protein samples were denatured by heating at 100°C for 3 min in sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 5% 2-ME and 0.002% bromophenol blue). Electrophoresis was carried out in a vertical slab gel apparatus (Bio-Rad, Richmond, CA, U.S.A.) at 80 V for 2 hr and then at 150 V for 4–5 hr. After electrophoresis, gel was stained with Coomassie blue R250, and dried gel was loaded for autoradiography at -70°C for 7 days.

FAST and FAST inhibition

Cockroach IgE FAST (3M Diagnostic System Inc., Santa Clara, CA, U.S.A.) was performed according to the manufacturer's instruction. Cockroach-IgE antibody of atopic subjects was determined [10], and four sera with high levels of specific-IgE antibody were pooled in equal volumes. As for the FAST inhibition, 30 µl of patients' sera mixture and 30 µl each of inhibitor or translation

Table 1. Translation of cockroach and TMV RNA in a rabbit reticulocyte system

RNA	Incorporation of ^{35}S -methionine*
TMV mRNA control	621767 (59326–64513)
Cockroach poly(A) ⁺	51045 (49125–53218)
Cockroach poly(A) ⁻	7325 (6912–7543)
None added	5364 (5123–5520)

* The values represent the mean of four experiments of the TCA-precipitated counts/min obtained with 1 μg of poly(A)⁺ and 5 μg of poly(A)⁻ cockroach RNA after 60 min incubation, and the range of counts/minute is indicated in parentheses.

mixtures were allowed to react with coated Cr-PI allergen overnight at 4°C. After incubation and washing, alkaline phosphatase labelled monoclonal anti-human IgE conjugate was added and incubated at room temperature for 2 hr. Excess enzyme-labelled anti-IgE was washed away and fluorescence substrate (4-methylumbelliferyl phosphate in 2-amino-2-methyl propanol buffer, pH 9.5) was then added into the well and incubated at room temperature for 40 min. After incubation, fluorescence signal units (FSU) were determined by a 3M Diagnostic System fluorometer. Inhibition was expressed as: $[1 - (b - c) / (a - c)] \times 100$, where a, FSU in the absence of inhibitor, b, FSU in the presence of inhibitor and c, FSU in the absence of a reaginic serum.

Results

Biologically active mRNA was isolated from the whole body of American cockroach as described under Methods and translated *in vitro* using a rabbit reticulocyte lysate system. Ten grams of cockroach yielded an average of 656 μg of total RNA, and the final yield was approximately 10.2 μg of mRNA. The efficiency of translation from cockroach poly(A)⁺ RNA was determined by the incorporation of ^{35}S -methionine (Table 1). In four experiments, an approximately 8- to 11-fold (average of 9.5-fold) increase in label incorporation over control was achieved with poly(A)⁺ RNA, whereas only a 1.36-fold increase in incorporation was obtained with poly(A)⁻ RNA as shown in Table 1.

The MWs of the translated cockroach proteins analysed by SDS-PAGE in combination with autoradiography were compared with control translated products as demonstrated in Fig. 1. Many cockroach proteins, ranging from 12 to 200 kD, were synthesized (Fig. 1, lane d), and only a faint and indistinct radiostaining band was

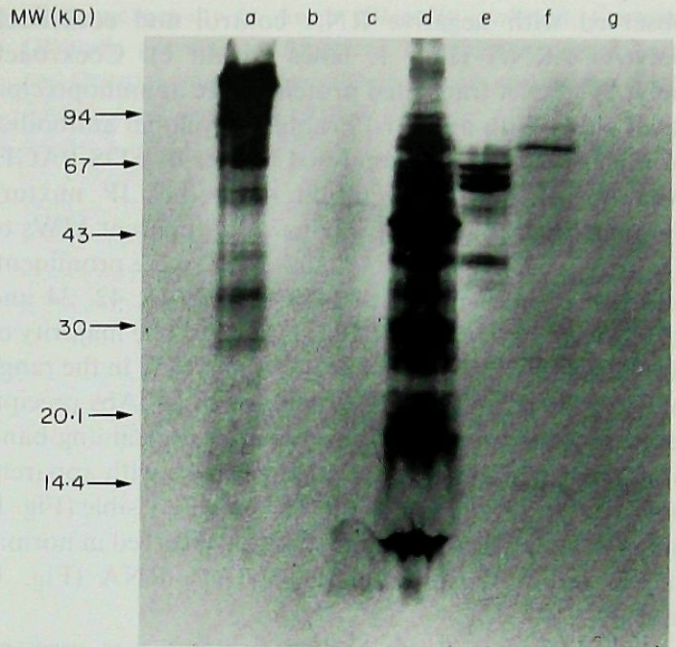


Fig. 1. Autoradiography of SDS-PAGE analysis of *in vitro* translated proteins. (a) TMV mRNA control (0.2 μl). (b) Negative RNA control (5 μl). (c) Cockroach poly(A)⁻ RNA (5 μl). (d) Cockroach poly(A)⁺ RNA (5 μl). (e) Rabbit polyclonal anti-Cr-PI IP mixtures (20 μl). (f) MoAbs anti-Cr-PI IP mixtures (20 μl). (g) Pre-immunized rabbit sera IP mixtures (20 μl). The apparent MWs (KD) of the protein markers are indicated on the left hand side of the autoradiograph.

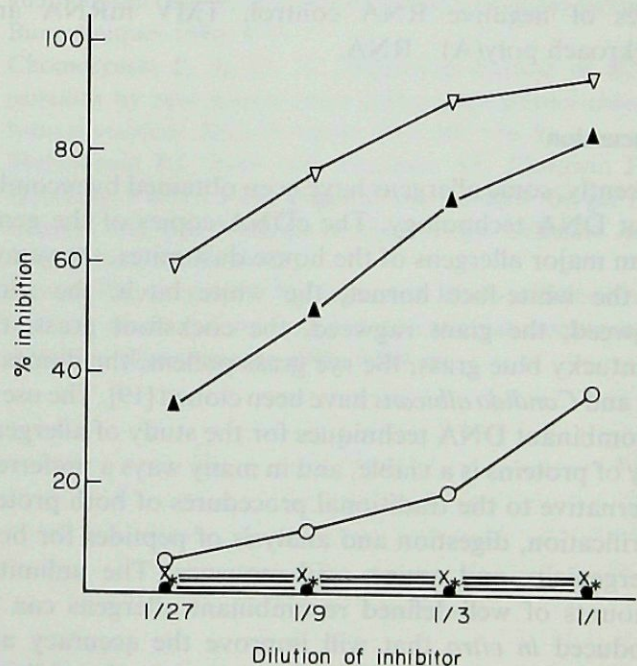


Fig. 2. Analysis of the inhibitory activity of CRa-A, Cr-PI and the translation mixtures by FAST with human reaginic serum pool. ∇ , CRa-A (200 $\mu\text{g}/\text{ml}$ of protein). $\blacktriangle$, Cr-PI (20 $\mu\text{g}/\text{ml}$ of protein). $\circ$, Cockroach poly(A)⁺ RNA. $\times$, Cockroach poly(A)⁻ RNA. $*$, TMV mRNA control. $\bullet$, Negative mRNA control.

observed with negative RNA control and cockroach poly(A)⁻ RNA (Fig. 1, lanes b and c). Cockroach poly(A)⁺ RNA translated proteins were immunoprecipitated either with anti-Cr-PI rabbit polyclonal antibodies or murine MoAbs and analysed further by SDS-PAGE. Analysis of polyclonal rabbit antibodies IP mixture demonstrated that nine proteins with apparent MWs of 78, 72, 65, 62, 60, 49, 42, 40, and 38 kD were prominent, and five components with MWs of 90, 45, 42, 34 and 26 kD were barely visible (Fig. 1, lane e). The majority of those immunoprecipitated proteins had MW in the range of 38 to 78 kD. SDS-PAGE analysis of MoAbs precipitated products revealed a prominent radiostaining band at a MW of 78 kD, and six other proteins with apparent MWs of 90, 72, 65, 45, 26 and 15 kD were visible (Fig. 1, lane f). No labelled protein band was observed in normal rabbit serum IP mixture of poly(A)⁺ RNA (Fig. 1, lane g).

The allergenicity of labelled proteins was assessed further by FAST inhibition using an appropriate atopic sera containing high levels of specific anti-cockroach IgE antibodies and compared with CrA-A and Cr-PI. Serial dilutions of inhibitors were assayed for their ability to inhibit the binding of IgE to Cr-PI, and results are shown in Fig. 2. The inhibition value obtained with highest concentration of CrA-A, Cr-PI and poly(A)⁺ RNA translation mixture was 96%, 92% and 38%, respectively; and no inhibition were observed from translation mixtures of negative RNA control, TMV mRNA and cockroach poly(A)⁻ RNA.

Discussion

Recently, some allergens have been obtained by recombinant DNA technology. The cDNA copies of the genes from major allergens of the house dust mites, the venom of the white-face hornet, the white birch, the short ragweed, the giant ragweed, the cocksfoot grass, the Kentucky blue grass, the rye grass pollens, the domestic cat and *Candida albicans* have been cloned [19]. The use of recombinant DNA techniques for the study of allergenicity of proteins is a viable, and in many ways a preferred, alternative to the traditional procedures of both protein purification, digestion and analysis of peptides for both allergenicity and amino acid sequence. The unlimited amounts of well-defined recombinant allergens can be produced *in vitro* that will improve the accuracy and reliability of *in vitro/in vivo* diagnostic tests. In addition, manipulating of allergen cDNA clones may provide information on size, structure, and allergenicity of allergens. Furthermore, epitopes of allergen-specific T- and B-cell clones can be identified, and the structurally modified allergens can be used to develop new forms of

immunotherapy. The purpose of this study is to isolate the biologically active mRNA from American cockroach and to analyse the *in vitro* translated products as a preliminary phase of a cDNA-based cloning strategy.

From many attempts with different methods [23–26], RNA was successfully extracted from the whole body of American cockroach using high concentrations of choropic salt guanidine isothiocyanate in the presence of 2-ME (Table 1). Polyadenylated mRNA was isolated by oligothymidilic acid-cellulose chromatography and translated in an *in vitro* system. Products of translation were analysed to determine whether the synthesized ³⁵S-methionine-labelled proteins were cockroach specific. In our previous study, nine components of MWs of 120, 110, 90, 78, 72, 49, 45, 29 and 26 kD which were able to bind specific human IgE as determined by immunoblot analysis have been identified as major or principal allergens of American cockroach [11]. In this study, combination of polyclonal and monoclonal antibodies IP mixtures revealed that at least 14 proteins with apparent MW from 15 to 90 kD were synthesized (Fig. 1, lanes e and f). Among those 14 proteins, six with MWs of 90, 78, 72, 49, 45 and 26 kD corresponded with previously identified allergens. Thus, the poly(A)⁺ RNA isolated from the whole body of American cockroach contained mRNA specific for proteins correlating to Cr-PI as determined by SDS-PAGE analysis. More importantly, allergenicity of synthesized proteins was defined by FAST inhibition studies with IgE antibodies of human reagenic serum pool (Fig. 2). The inhibition of 38% was obtained with the highest concentration of poly(A)⁺ RNA translation mixture, and required approximately 0.028 µg of this protein as determined from Cr-PI inhibition curve by extrapolation. Concentration of 6.0 µg and 0.6 µg was required to obtained 96% and 92% inhibition with CrA-A and Cr-PI, respectively.

In conclusion, results obtained in these studies indicated that it is possible to isolate biologically active mRNA from the whole body of American cockroach. Thus, the presence of mRNA coding for Cr-PI is encouraging and raised the possibility of introducing cockroach cDNA genes in *Escherichia coli*. Cloning of cDNA coding for principal and major allergens of American cockroach, and analysis/characterization of Cr-PI- and/or specific IgE-positive clones are currently in progress in our laboratory.

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References

- 1 Marchand AM. Allergy to cockroach. Bol Assoc Med Puerto Rico 1966; 58:49-53.
- 2 Bernton HS, Brown H. Cockroach II: the relation of infestation to sensitization. South Med J 1967; 60:852-5.
- 3 Schulaner FA. Sensitivity to the cockroach in three groups of allergic children. Pediatrics 1970; 45:465-6.
- 4 Mendoza J, Synder FD. Cockroach sensitivity in children with bronchial asthma. Ann Allergy 1970; 28:159-63.
- 5 Bernton HS, McMahon TF, Brown H. Cockroach asthma. Br J Dis Chest 1972; 66:61-6.
- 6 Kang B. Study on cockroach antigens as probable causative agent in bronchial asthma. J Allergy Clin Immunol 1976; 58:357-65.
- 7 Twarog RJ, Picone FJ, Strunk RS, So J, Colten HR. Immediate hypersensitivity to cockroach. J Allergy Clin Immunol 1977; 59:154-60.
- 8 Kang B, Sulit N. A comparative study of prevalence of skin hypersensitivity to cockroach and house dust antigens. Ann Allergy 1978; 41:333-6.
- 9 Kang B, Vellody D, Homburger H, Yunginer JW. Cockroach cause of allergic asthma. Its specificity and immunologic profile. J Allergy Clin Immunol 1979; 63:80-6.
- 10 Lan JL, Lee DT, Wu CH, Chang CP, Yeh CL. Cockroach hypersensitivity: preliminary study of allergic cockroach asthma in Taiwan. J Allergy Clin Immunol 1988; 82:736-40.
- 11 Wu CH, Lan JL. Cockroach hypersensitivity: isolation and partial characterization of major allergens. J Allergy Clin Immunol 1988; 82:727-35.
- 12 Stankus RP, O'Neil CE. Antigenic/allergenic characterization of American and German cockroach extracts. J Allergy Clin Immunol 1988; 81:563-70.
- 13 Schou C, Lind P, Fernandez-Caldas E, Lockey RF, Lowenstein H. Identification and purification of an important cross-reactive allergen from American (*Periplaneta americana*) and German (*Blattella germanica*) cockroach. J Allergy Clin Immunol 1990; 86:935-46.
- 14 Kang BC, Wilson M, Price KH, Kambara T. Cockroach-allergen study: allergen patterns of three common cockroach species probed by allergic sera collected in two cities. J Allergy Clin Immunol 1991; 87:1073-80.
- 15 Helm RM, Squillace DJ, Jones RT, Brenner RJ. Shared allergenic activity in Asian (*Blattella asahinai*), German (*Blattella germanica*), American (*Periplaneta americana*), and Oriental (*Blatta orientalis*) cockroach species. Int Arch Allergy Appl Immunol 1990; 92:154-61.
- 16 Lehrer S, Horner WE, Menon P, Stankus RP. Comparison of cockroach allergenic activity in whole body and fecal extracts. J Allergy Clin Immunol 1991; 87:574-80.
- 17 Pollart SM, Mullins DE, Vailes LD *et al.* Identification, quantitation, and purification of cockroach allergens using monoclonal antibodies. J Allergy Clin Immunol 1992; 87: 511-21.
- 18 Wu CH, Chiang BT, Fann MC, Lan JL. Production and characterization of monoclonal antibodies against major allergens of American cockroach. Clin Exp Allergy 1990; 20: 675-81.
- 19 Scheiner O. Recombinant allergens: biological, immunological and practical aspects. Int Arch Allergy Immunol 1992; 98:93-6.
- 20 Maniatis T, Fritsch EF, Sambrook J. Spectrophotometric determination of DNA or RNA. In: Molecular cloning: a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory, 1982:468.
- 21 Olliver CL, Boyd CD. Immunoprecipitation of *in vitro* translation products with protein A bound to Sepharose. In: Walker JM, ed. Methods in molecular biology: nucleic acids. New York: Academic Press, 1984:157-60.
- 22 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-5.
- 23 Turpen TH, Griffith MO. Rapid isolation of RNA by a guanidinium thiocyanate/cesium chloride gradient method. Biotechniques 1986; 4:11-5.
- 24 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156-9.
- 25 MacDonald RJ, Swift GH, Przybala AE, Chirgwin JM. Isolation of RNA using guanidinium salts. In: Berger SL, Kimmel AR, eds. Methods in enzymology. Orlando: Academic Press, 1987:219-22.
- 26 Briscoe PR, Jorgensen TJ. Improved RNA isolation from cells in tissue culture using a commercial nucleic acid extractor. Biotechniques 1991; 5:594-6.