Optimal conditions for the storage of German cockroach extract

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Abstract. Allergen extracts are commonly utilized for diagnosis and immunotherapy; however, the stability of protease-rich extracts is important for a precise diagnosis and treatment efficacy. The present study determines the optimal conditions for the storage of German cockroach allergen extract. Cockroach extracts were reconstituted in four buffers: normal saline (NS), 50% glycerol in NS, 0.3% phenol in NS, or 0.3% phenol and 50% glycerol in NS. The extracts in different buffers were stored either at room temperature (18-26˚C, RT) or refrigerated (2-8˚C). Subsequently, the protein concentration and allergen content (Bla g 1 and Bla g 2) in the extracts were examined for the course of one year. Extract potency was estimated by inhibition ELISA. At least 90.5% protein, 94.4% Bla g 1, 65.2% Bla g 2, and 91.4% potency remained after one year when 50% glycerol NS was added to the extract with refrigeration. However, less than 13.7% protein, 17.1% Bla g 1, 0% Bla g 2 and 32.5% potency were maintained after one year when 50% glycerol NS was not added to the extract and was maintained at RT. The addition of 0.3% phenol NS did not show significant effects on extract stability. The addition of 50% glycerol NS and refrigerated storage temperature were found to be important factors for increasing the shelf life of protease-rich cockroach extract.

Introduction

Cockroach sensitization is a risk factor for asthma. Protease-activated receptors (PAR)-2, Toll-like receptors (TLRs) and C-type lectin receptors have been suggested to play a role in the penetration of cockroach allergens through epithelial cells and allergic inflammation (1). Strong protease activity in cockroach extracts can degrade protein components within the extract. Dilution and mixture of cockroach extract with pollen extracts was reported to cause a loss of allergen potency (2). The additions of protease-inhibitor (ε-aminocaproic acid) and sucrose were reported to increase the shelf life of the protease-rich American cockroach extract (3); however, the potential side effects of this protease inhibitor have not been examined.

More recently, mixtures of cockroach allergens with other indoor allergens for immunotherapy have been found useful when more than 50% glycerol is included, even though current immunotherapy guidelines recommend the separation of high protease extracts from other products if possible (4,5). Moreover, cockroach extracts for immunotherapy are currently not standardized since allergen and protein contents in commercial extracts vary greatly, and the potency of commercial extracts was estimated as 10 to 8,570 bioequivalent units (BAU)/ml (6).

In the present study, we determined the optimal storage conditions for protease-rich German cockroach extracts prepared in a standardized manner using a Korean isolate (7). The changes in protein and major allergen (Bla g 1 and Bla g 2, respectively) contents as well as allergen potency (total IgE reactivity) in the extract stored under various conditions were examined over a 1-year time period.

Materials and methods

Serum samples. Serum samples were collected from 15 patients (age range 14-54 years, mean 32 years, 9 males and 6 females) at the Allergy-Asthma Center at Severance Hospital (Seoul, Korea) between November 2014 and December 2018. Patient consent was obtained before the blood collection. Specific IgE to German cockroach was determined using the ImmunoCAP system (Thermo Fisher Scientific, Inc.) and serum samples with specific IgE levels higher than 3.5 kU_A/l were selected. A pooled serum sample from 13 healthy subjects (age range 25-81 years, mean 48 years, 9 males and 10 females) was used as a negative control. samples were taken between March 19, 2014 and May 6, 2016 at Severance Hospital, Seoul, Korea. The Institutional Review Board of the Yonsei University Health System approved this study (no. 4-2013-0397).

Allergen extraction. Allergen extract was prepared as previously described (7). In brief, lyophilized cockroach was homogenized in liquid nitrogen, defatted with ethyl ether,

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extracted in phosphate-buffered saline (PBS), dialyzed against distilled water (DW), filtered (0.22 μm) and lyophilized again. The protein concentration was determined by Bradford assay (Bio-Rad Laboratories) after dissolution in appropriate buffers.

The lyophilized extract was reconstituted in one of four solutions: normal saline (NS), 50% glycerol in NS, 0.3% phenol in NS, or 0.3% phenol and 50% glycerol in NS. The dissolved extracts were aliquoted and maintained at room temperature (RT, 18-26˚C) or refrigerated (2-8˚C). Samples were taken for testing at weeks 1, 2, 4, 9, 13, 26 and 52.

Protein analyses and measurement of allergen content. Protein concentration was measured via Bradford assay using bovine serum albumin (BSA) as the standard. Allergen content (Bla g 1 and Bla g 2) was assessed using two-site ELISA (Indoor Biotechnologies Inc.).

IgE antibody binding inhibition assay. Allergen extract potency was compared using inhibition ELISA. Allergen extract (100 μl) at week 0 was coated overnight at 4˚C at a 10 μg/ml concentration in 50 mM carbonate buffer, pH 9.6. Simultaneously, 4-fold diluted pooled serum from German cockroach allergy patients (n=15) was pre-incubated overnight at 4˚C with the extract of interest (1/10 volume) or 1% BSA solution as a control. IgE antibodies were detected with biotinylated goat anti-human IgE (ε chain specific; Vector Laboratories Inc.) and streptavidin-peroxidase (Sigma-Aldrich). The inhibition percentage was calculated as $(1-A_i/A_0)$ x100, where A_i indicates the absorbance at 450 nm with an inhibitor and A_0 indicates the absorbance without an inhibitor.

SDS-PAGE and IgE immunoblotting. Protein profile and IgE reactive components were examined by SDS-PAGE and IgE immunoblotting. Proteins (20 μl) were separated on 12% SDS-PAGE gels under reducing conditions. Gels were stained with Coomassie brilliant blue or transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Waters & Process Technologies). IgE reactive components were probed with alkaline phosphate conjugated goat anti-human IgE (1:1,000-dilution) (ε chain specific; Sigma-Aldrich), and color development was conducted with nitro blue tetrazolium and 3-bromo-4-chloro-5-indolyl-phosphate (Promega).

Statistical analyses. Statistical differences between test groups were analyzed by two-way ANOVA followed by Bonferroni correction. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Protein content in German cockroach extract. Initial protein concentration was determined to be 0.4 mg/ml. Approximately 33.8% of the original protein concentration was detected after 52 weeks when 50% glycerol was added to the extract in NS, whereas 4.1% remained in the extracts of NS at RT (Fig. 1A). When refrigerated, >90.0% of the protein was detected when 50% glycerol NS was added, while <78.0% was detected without glycerol (Fig. 1B).

Allergen content in German cockroach extract. Initial concentrations of Bla g 1 and Bla g 2 were 35.3 and 7.0 μg/ml, respectively. Bla g 1 content in the extract stored at RT remained at 17.1% in NS, 7.0% in 0.3% phenol NS, 100% in 50% glycerol NS, and 97.1% in glycerol phenol NS (Fig. 2A). Bla g 1 content detected in the refrigerated extracts was 96.4% in NS, 100% in 50% glycerol NS, 88.0% in 0.3% phenol NS and 94.4% in glycerol with phenol NS (Fig. 2B). A similar pattern of Bla g 2 content was observed, even though Bla g 1 in the extract was shown to be more stable than Bla g 2 (Fig. 2C and D).

Allergen potency of German cockroach extract. Potency was retained at 73.0% in 50% glycerol NS and 66.9% in 50% glycerol with phenol NS when stored at RT, whereas 26.9% in NS and 32.5% in phenol NS were retained in the absence of glycerol (Fig. 3A). When refrigerated, 91.4% potency in 50% glycerol NS and 92.6% potency in 50% glycerol with phenol NS of potency was maintained, while 72.9% of the allergen potency in NS and 74.9% in phenol NS were retained in the absence of glycerol (Fig. 3B).

Change of protein profile and IgE reactive components. Notably, a 35-kDa component and components >55 kDa were shown to be degraded when the extracts were maintained at RT (Fig. 4A). Slow disruption of the 35 kDa band was observed in the refrigerated extract in 50% glycerol NS and also bands around 70 kDa in the refrigerated extract in 0.3% phenol NS (Fig. 4B). Notably, some partially degraded proteins produced bands of ~55 kDa in 50% glycerol NS at RT, 50% glycerol with 0.3% phenol NS at RT, and 50% glycerol with 0.3% phenol NS and refrigerated, ~38 kDa in 0.3% phenol NS at RT.

Strong IgE reactions were detected between 55 and 100 kDa components when extracts were dissolved in a solution with 50% glycerol NS (regardless of 0.3% phenol) and kept refrigerated (Fig. 4B, C and D). IgE reaction to these components seemed to correlate with potency. IgE reactivity to the 34 kDa band was detected only in the refrigerated samples and within the first few weeks of the sampling at RT (Fig. 4C and D).

No IgE reactive component was detected by IgE immunoblotting with the extracts at week 0, which should include all the allergenic components (Fig. 4E and G), using a pooled serum sample with healthy subjects (Fig. 4F and H).

Discussion

Commercial cockroach extracts are highly variable in terms of allergen content and potency. However, high protease activity in cockroach extracts makes it more difficult to standardize the allergen extracts. Glycerol is a well-known stabilizer of allergen extracts (8). However, stability of German cockroach extract has not been described. Therefore, we aimed to observe what occurs with German cockroach extract which has strong protease activity. In this study, the protein concentration was well conserved when refrigerated, and the addition of 50% glycerol NS seemed to inhibit the protease activity and enhanced cockroach extract stability.

The change of IgE reactive components were investigated by SDS-PAGE and IgE immunoblotting. However, it was not certain concerning the identity of each protein band. It seems that a high salt concentration affects the solubility of various

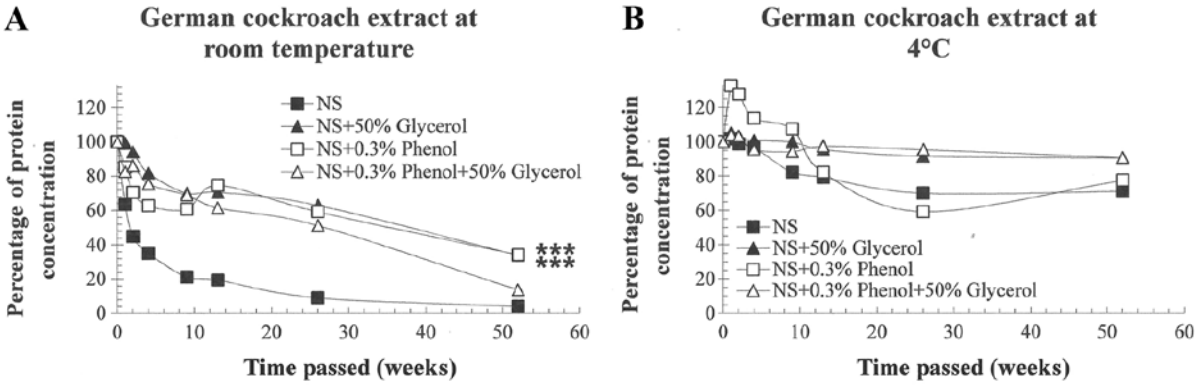

Figure 1. Change of protein concentration in cockroach extract over one year of storage under various conditions. Protein concentration was determined using the Bradford assay in the extracts kept at (A) room temperature and (B) refrigerated. ***P<0.001. NS, normal saline.

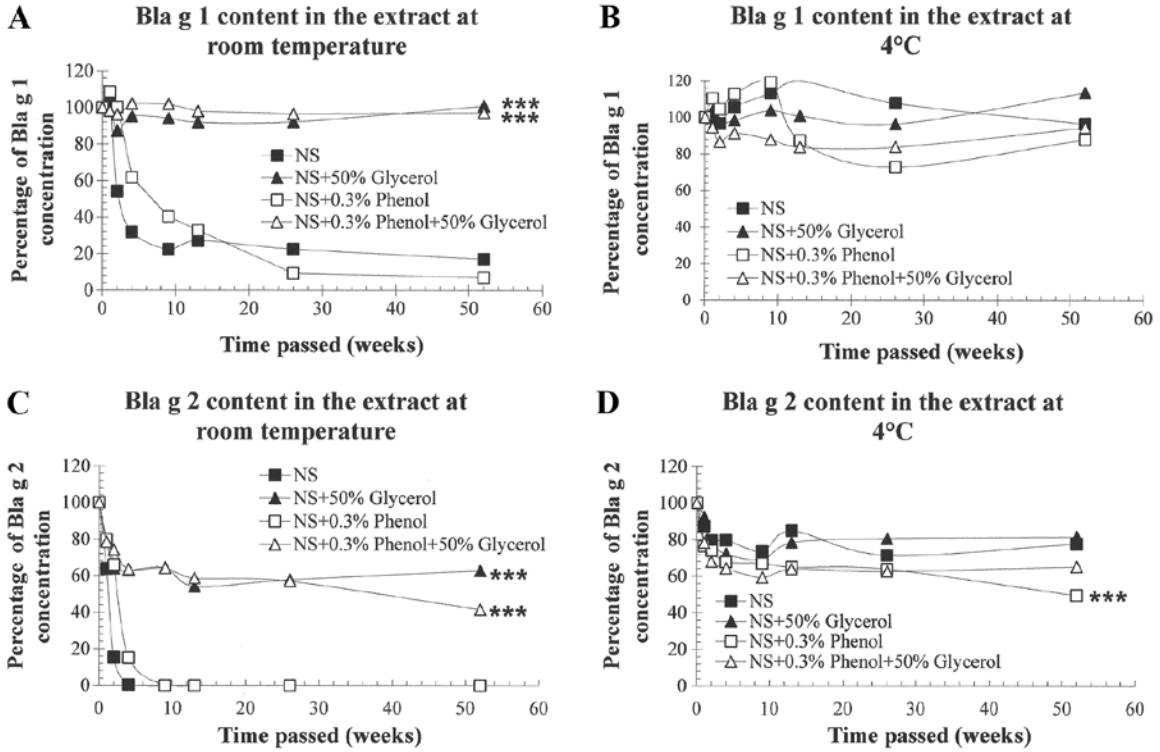

Figure 2. Change of Bla g 1 and Bla g 2 content in cockroach extract over one year of storage at various conditions. Bla g 1 content in the extract stored at (A) room temperature and (B) refrigerated, Bla g 2 content at (C) room temperature, and (D) refrigerated, measured by a two-site ELISA. ***P<0.001. NS, normal saline.

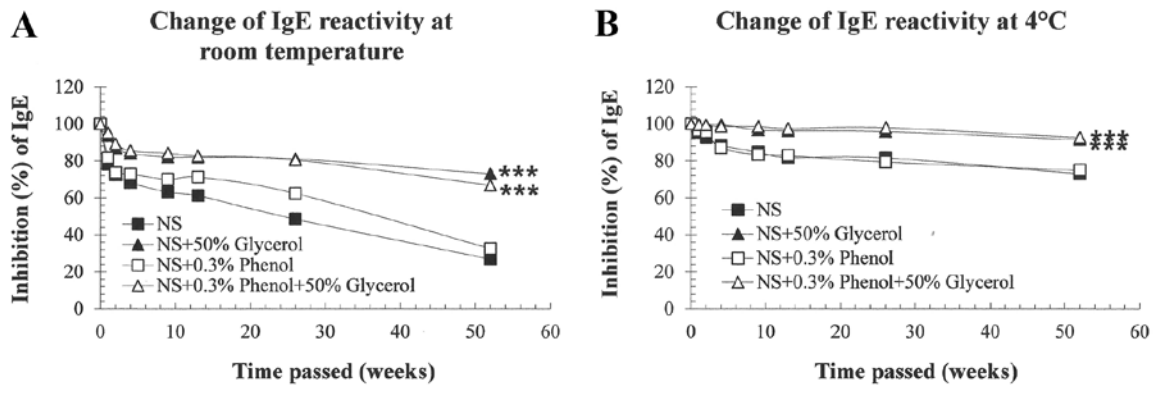

Figure 3. Change of extract potency over one year of storage under various conditions. IgE reactivity of the extracts was compared with the initial state by inhibition ELISA. Cockroach extracts were kept at (A) room temperature and (B) refrigerated. ***P<0.001. NS, normal saline.

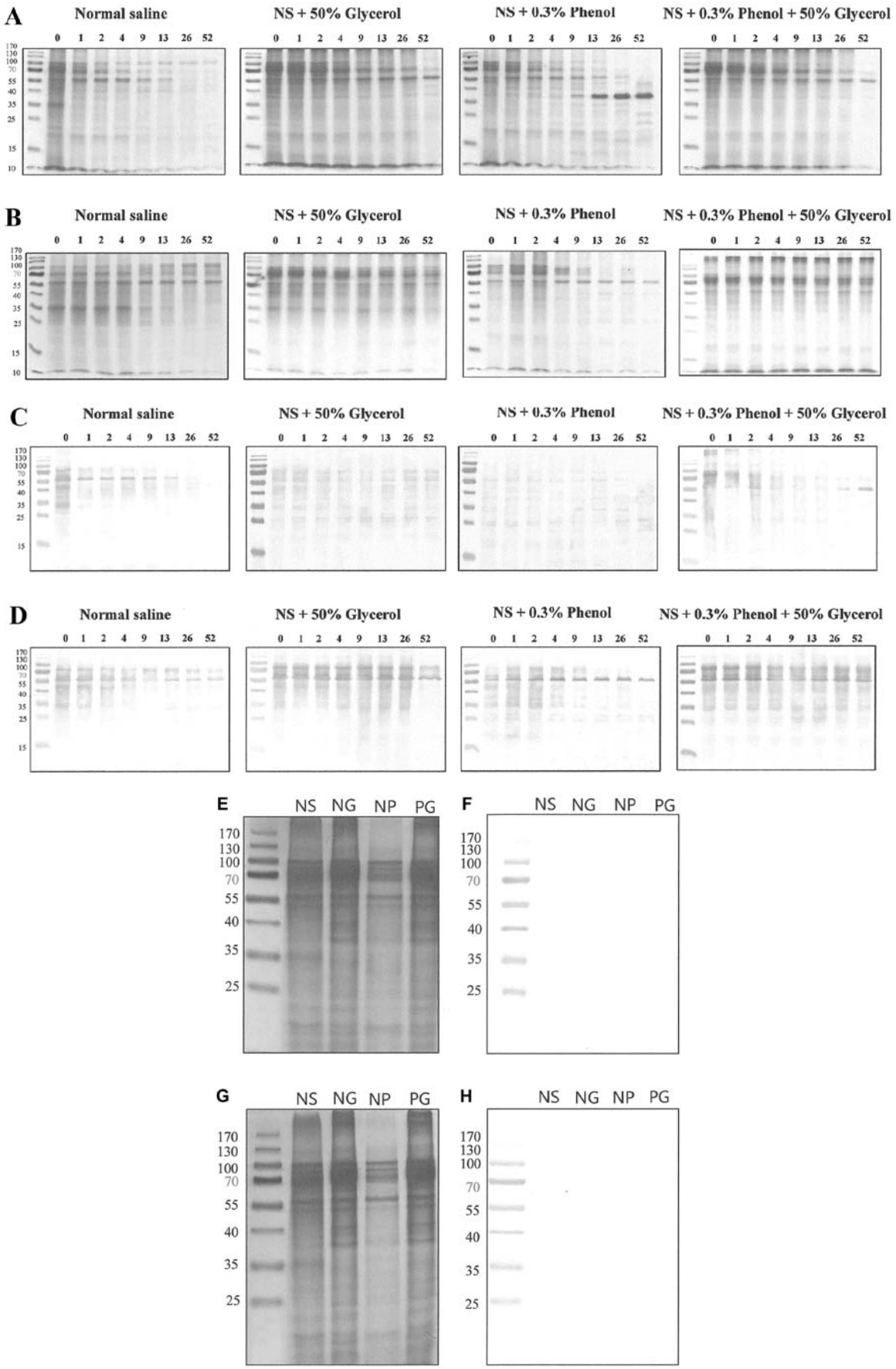

Figure 4. Change of protein profile and IgE reactive components over one year of storage in various conditions. SDS-PAGE of the extracts stored at (A) room temperature and (B) refrigerated. IgE immunoblotting of the extracts maintained at (C) room temperature and (D) refrigerated. The extracts stored at room temperature and refrigerated at week 0 were also examined by (E and G) SDS-PAGE and (F and H) IgE immunoblotting using a pooled serum sample from the healthy subjects as negative controls. NS, normal saline; NG, 50% glycerol in normal saline; NP, 0.3% phenol in normal saline; PG, 0.3% phenol and 50% glycerol in normal saline.

components in the extracts and produces high molecular weight bands in SDS-PAGE. However, these components were not allergenic, as examined by IgE immunoblotting. Furthermore, phenol, which is a reagent commonly used to eliminate protein in a laboratory, also influenced the banding pattern by SDS-PAGE. Some protein degradation by phenol may have caused the different protein profile and IgE reaction. The phenol effect is thought to be minimized by the addition of glycerol. In this study, Bla g 1 and Bla g 2 content in the cockroach extract were examined because two-site ELSIA kits for these allergens are commercially available. However, a growing body of evidence suggests that Bla g 1 may not be a major allergen. None of the cockroach allergens identified to date appear to be immunodominant (6,9,10). In recent studies, Bla g 5, Bla g 11 and Bla g 2 were determined as relatively important (6,11). A more convenient and cheap assay for the quantification of Bla g 5 and Bla g 11 is required. Moreover, partially degraded allergens could retain IgE epitopes, although allergens could not be quantified with the two-site ELISA that recognizes two different epitopes. Liquid chromatography and multiple reaction monitoring mass spectrometry (LC-MRM MS) may offer rapid and accurate quantification of allergens. However, current LC-MRM MS technology is unlikely to detect and reflect the change in conformational epitopes in the extracts (12).

Extract potency is thought to better reflect extract reactivity stability for allergic reactions that are elicited by allergen-specific IgE antibodies. In general, potency was well preserved when 50% glycerol NS was added and refrigerated, as protein and allergen content were preserved. Notably, slower deterioration of potency was observed compared to the protein and allergen contents, reflecting the fact that some of the partially degraded allergens retained IgE reactivity.

Abrupt deterioration of cockroach extract in the absence of 50% glycerol NS is in line with the analyses of commercial aqueous and glycerinated cockroach extracts (13). On the other hand, the commonly used bacteriostatic agent phenol seems to have little effect on the stability of cockroach extract, although it has been shown to be disadvantageous over time with some pollen extracts (14).

The present study has several limitations. First, the effect of human serum albumin (HSA), a common reagent used as a stabilizer for allergen dilution, was not included. HSA is commonly added to protect allergen adsorption to glass vials and protein digestion. However, HSA is known to be effective in diluted extracts. Second, temperature excursions during shipping and clinical use were not tested; therefore, the results could be applied to the concentrated extract but not to diluted extracts. Finally, the change of protease activity over time was not examined. Proteases can elicit inflammatory reactions; however, allergenicity is not determined by protease activity but instead by IgE reactivity.

Taken together, cockroach extract with high protease activity can be stored for at least one year by the addition of 50% glycerol NS and refrigeration. The present study could facilitate the further refinement of allergen extract standardization with high protease activity and the development of improved diagnostics and immunotherapeutics. There is still great need for a surrogate system to assess the quality of cockroach extract.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KYJ drafted the manuscript. JL and JEY performed SDS-PAGE and IgE immunoblotting experiments. KHP, JHL and JWP collected serum samples. JDK prepared and provided cockroach extracts. KYJ, KHP, JHL and JWP designed the study and experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Patient consent was obtained before the blood collection. The Institutional Review Board of the Yonsei University Health System approved this study (no. 4-2013-0397).

Patient consent for publication

Not applicable.

Competing interests

KYJ, KHP, JHL, JDK and JWP have stocks in Prolagen. KYJ receives a consultancy fee from Prolagen.

Authors' information

KYJ is a technical advisor, JDK is a CEO, and JWP is a CTO of Prolagen.

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