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The Effects of Storage Conditions on the Stability of House Dust Mite Extracts

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Purpose: Allergen extracts from the house dust mite (HDM, *Dermatophagoides pteronyssinus*) are widely utilized for diagnosis and treatment of allergic diseases. It is known that allergen extracts degrade and lose potency when stored over time. **Methods:** This study aimed to determine the optimal conditions for stability of allergen extracts. This study was undertaken to investigate the optimal storage conditions for HDM extracts, the effects of adding 0.03% human serum albumin (HSA) and 50% glycerol were evaluated at -20°C, 4°C, and room temperature (RT). Changes in protein and group 1 major allergen (Der p 1) concentration, as well as allergenicity were measured over a 1 year period using the Bradford assay, two-site ELISA, and ELISA inhibition. **Results:** Protein concentrations decreased by 86%, 51%, and 6% at RT, 4°C, and -20°C, respectively, when stored in distilled water. Overall allergenicity remained high (89.9%) when the extracts was reconstituted in 50% glycerol solution, and was 93.1% when reconstituted in 50% glycerol and 0.03% HSA at RT. Allergenicity was decreased to 36.6% and 33.3%, however, reconstitution in DW or 0.03% HSA solution at RT, respectively. Allergenicity was remained high as 92.0%-97.0% when stored at 4°C regardless of the buffer conditions. **Conclusions:** Storage temperature is the most important factor in preserving allergenicity of HDM extracts, which is ideal at 4°C. The addition of 50% glycerol to the storage buffer was also found to play an important role in increasing the shelf-life of HDM extracts at RT.

Key Words: Allergen; house dust mite; stability

INTRODUCTION

The house dust mite (HDM) is a major cause of respiratory allergic diseases.[1,2] Allergen extracts are standardized in the production of diagnostic and immuotherapeutic reagents,[3] and should ideally remain stable from the time of initial preparation to the time of clinical application. HDM extracts have been shown, however, to contain various proteases which could degrade proteins and decrease allergenic potency during storage.[4] Four groups of HDM allergens (1, 3, 6, and 9) are proteolytic enzymes.

Pollen extracts are known to be stable for 3-15 months at 4°C when dissolved in phosphate buffered saline (PBS) containing 50% glycerol.[5-8] Reports on the stability of HDM extracts, on the other hand, have not been consistent. Bousquet et al. (1985) showed that *D. pteronyssinus* extracts reconstituted in 50% glycerol loses its allergenic activity as measured by skin test titration.[8] They suggested that *D. pteronyssinus* extracts should not be used more than one month after reconstitution. In that study, the extracts were placed at room temperature (RT) for 1 h every day to mimic the natural conditions of allergen extracts usage. This temperature change is thought to greatly influence the stability of the extracts, however. Naerdal and Vilsvik (1983) reported that *D. farinae* extracts are stable for 8 months at 4°C if reconstituted in saline solution containing 0.03% human serum albumin (HSA).[9] HSA is believed to prevent adsorption of allergens into glass surfaces, especially in diluted allergen preparations.

In this study, we assessed the stability of HDM (*D. pteronyssinus*) extracts prepared using Korean HDM isolates.[10] Changes in protein content, Der p 1 concentration, and IgE reactivity were measured at -20°C, 4°C, and RT over the course of 12 months.

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MATERIALS AND METHODS

Mite extracts

D. pteronyssinus extract was prepared as previously described using whole bodies of cultured mites in bicarbonate buffer (pH 8.0).[10] The extract was subsequently dialyzed extensively against distilled water and lyophilized.

Storage of mite extracts

The lyophilized extracts was dissolved in one of four solutions including distilled water (DW), 50% glycerol, 0.03% HSA, or 0.03% HSA in 50% glycerol. The samples were aliquoted and stored at -20°C, 4°C, and RT (~26°C). The samples were withdrawn and tested at weeks 1, 2, 4, 9 (2 months), 13 (3 months), 26 (6 months), and 52 (1 year).

Measurements of protein and Der p 1 content

Protein concentrations were measured using the Bradford assay (Bio-rad, Hercules, CA, USA) with bovine serum albumin (BSA) as the standard. The content of Der p 1 in the extracts was assessed using a two-site ELISA kit (Indoor Biotechnologies Inc., Charlottesville, VA, USA).

Inhibition ELISA

Overall allergenicity of mite extracts was determined by inhibition ELISA. Each well was coated overnight at 4°C with 100 µL of 10 µg/mL *D. pteronyssinus* extracts in a 0.05 M sodium carbonate buffer (pH=9.6). The 1:1 serum dilutions (pooled sera from 5 subjects) were pre-incubated with various concentrations of inhibitors. IgE antibodies were subsequently detected using biotinylated goat anti-human IgE (epsilon chain specific; Vector, Burlingame, CA, USA) and streptavidin-peroxidase (Sigma-Aldrich, Sydney, Australia). The percent inhibition was calculated as $(1-A_i/A_0)\times100$, where A_0 indicates the absorbance at 450 nm with an inhibitor, and A_0 indicates the IgE value without an inhibitor.

SDS-PAGE and IgE immunoblotting

Proteins (35 µL of each sample) were separated on 12% SDS-polyacrylamide gels under reducing conditions. Gels were stained with Coomassie brilliant blue or transferred onto polyvinylidine difluoride (PVDF) membranes (0.45 µm, GE Waters & Process Technologies, Trevose, PA, USA). The membranes were reacted with a pooled serum sample (1:4 dilution) from 5 mite-allergic patients and incubated with a 1:1,000 dilution of alkaline phosphate conjugated goat anti-human IgE (ε-chain specific, Sigma-Aldrich, Sydney, Australia). Color was developed using nitroblue tetrazolium and 3-bromo-4-chloro-5-indolyl-phosphate (Promega, Madison, WI, USA).

RESULTS

Effects of storage conditions on protein concentration

The initial protein concentration of the extracts was determined to be 467.3 µg/mL. An abrupt decrease in protein concentration was observed within 3 weeks when stored at RT, though the addition of 50% glycerol to the storage buffer was shown to protect against protein degradation. More than 40% of the original

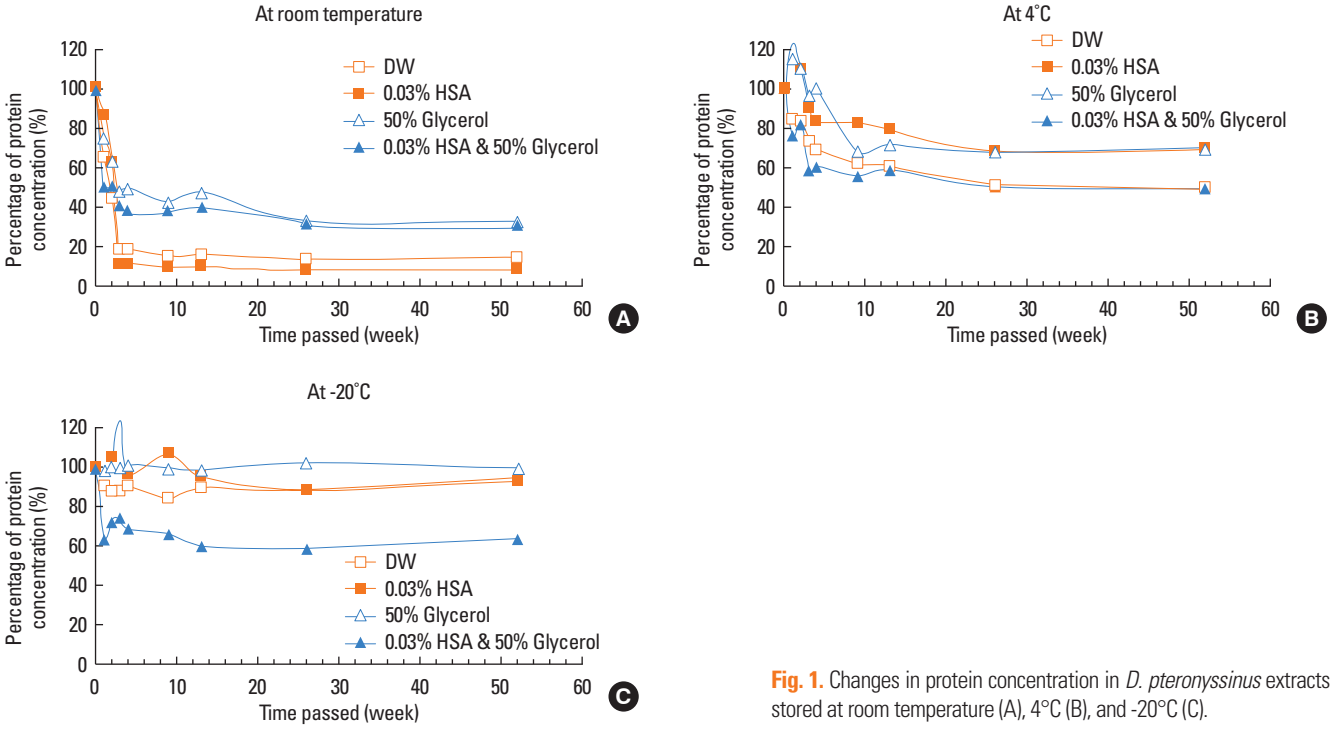

Fig. 1. Changes in protein concentration in *D. pteronyssinus* extracts stored at room temperature (A), 4°C (B), and -20°C (C).

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protein concentration was detected when 50% glycerol was added to the extracts, whereas less than 20% remained in the extracts without 50% glycerol (Fig. 1). At 4°C, the addition of 0.03% HSA or 50% glycerol played a protective role. At week 13, more than 70% of the original protein content was detected when HSA or glycerol had been added, while 60% of the protein was detected in the DW only storage condition. At -20°C, more than 80% of the protein remained intact for 1 year with or without HSA or glycerol. Interestingly, protein concentrations decreased only when both HSA and glycerol were included in the extracts stored at -20°C, with almost 40% of the protein content being lost between weeks 1 and 52.

Effects of storage conditions on Der p 1 concentration

The initial Der p 1 concentration was determined to be 2.15 ng/mL. Der p 1 concentration decreased under all conditions tested when stored at RT (Fig. 2). About 65% and 50% of initial Der p 1 content was detected at weeks 9 and 26, respectively, in all buffer conditions. Der p 1 content was found to be stable when stored at 4°C regardless of buffer composition. The addition of HSA, however, was shown to play a further protective role. At week 52, 79.3% and 73.7% of the original Der p 1 content was detected when the extract was reconstituted in DW or 50% glycerol solution, whereas 92.9% and 86.8% was detected when reconstituted in the solutions containing 0.03% HSA or 0.03% HSA and glycerol. Notably, at least 95% of the initial Der p 1 was detected when stored at -20°C at week 52 regardless of buffer composition.

Effects of storage conditions on IgE reactivity

The extracts reconstituted in 50% glycerol solution retained 89.9% (glycerol only) and 93.1% (glycerol and HSA) of IgE reactivity at week 52 at RT, whereas the extracts reconstituted without glycerol showed 33.3% (DW) and 36.6% (HSA only) reactivity at week 52 under RT conditions (Fig. 3). The extracts kept at 4°C showed no significant decreases in overall allergenicity regardless of buffer conditions, with 92.0%-97.0% of IgE reactivity remained under these conditions.

Effects of storage conditions on protein profiles

Changes in protein and allergen profiles were investigated using SDS-PAGE and IgE immunoblotting (Fig. 4). A protein with a molecular mass of ~15 kDa, likely a putative Der p 2, was found to be the strongest allergen. A ~42 kDa protein was the second strongest allergen, which showed quicker degradation over time at 4°C, compared to the 15 kDa allergen.

DISCUSSION

The stability of allergen extract is important for the diagnosis and treatment of allergic diseases. HDM extracts have been shown to contain various enzymes including proteases,[4] which result in protein degradation and reduced allergenic potency of the extracts.[11] Storage conditions and the addition of preservatives help prevent protein degradation and increase the shelf-life of the extracts. In this study, we examined the effects of buffer composition and temperature on the stability of *D. pteronyssinus* extracts.

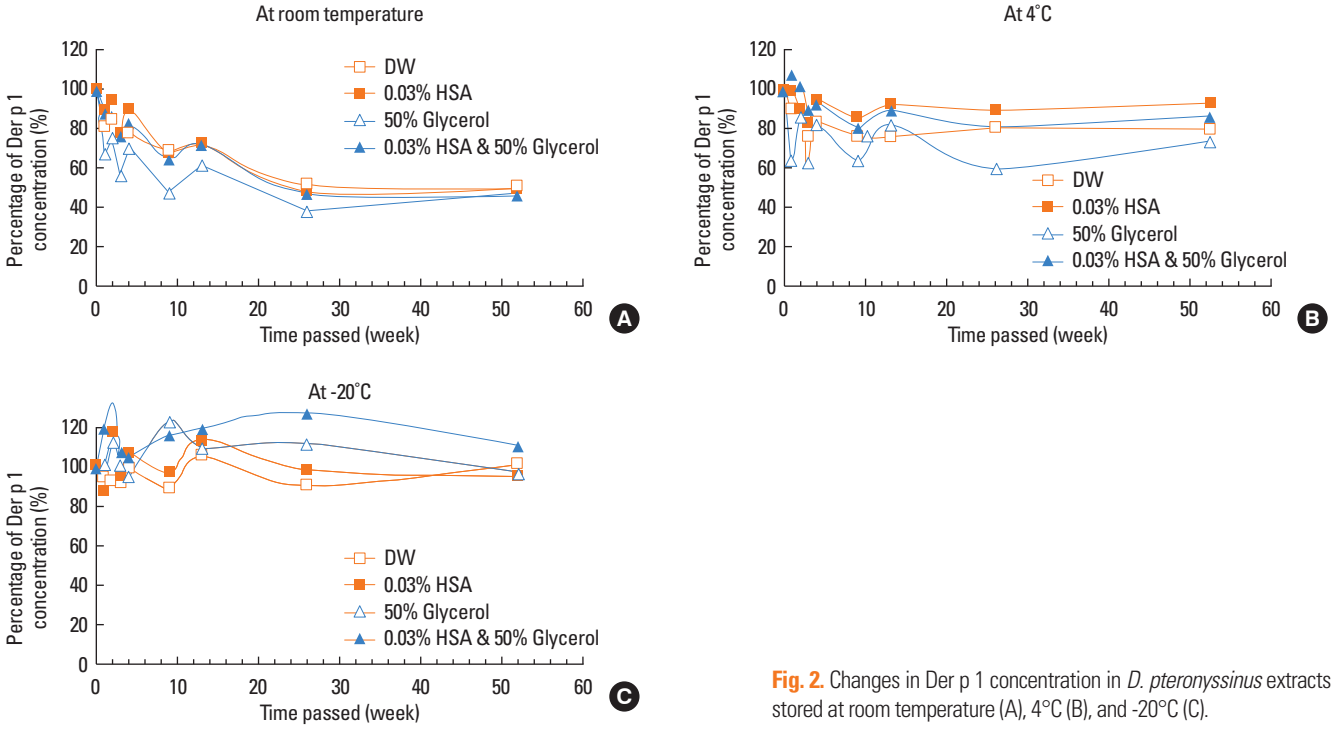

Fig. 2. Changes in Der p 1 concentration in *D. pteronyssinus* extracts stored at room temperature (A), 4°C (B), and -20°C (C).

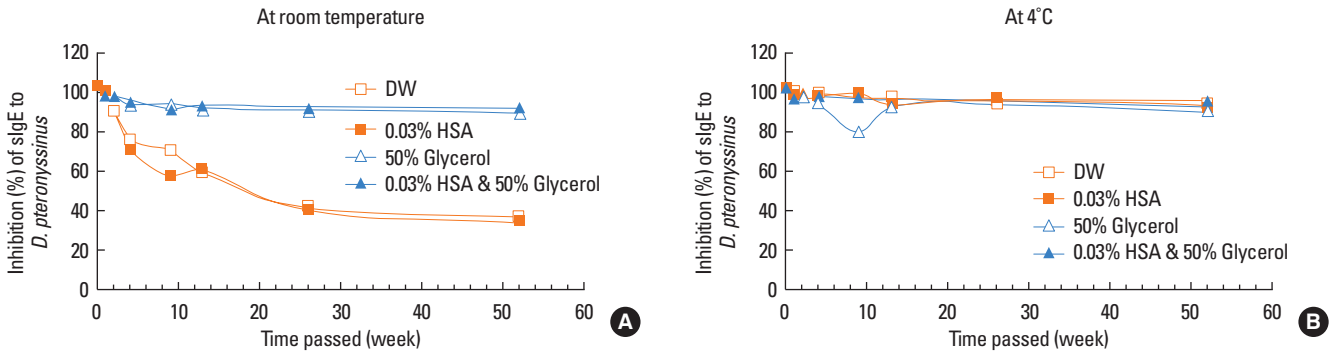

Fig. 3. Changes in allergenicity of *D. pteronyssinus* extracts stored at room temperature (A) and 4°C (B).

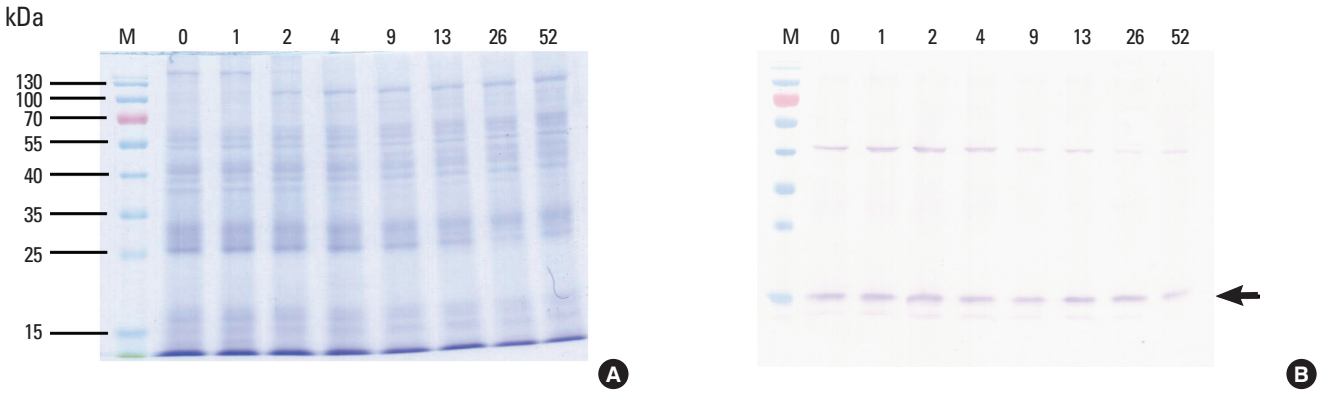

Fig. 4. SDS-PAGE profile (A) and IgE reactive bands (B) of *D. pteronyssinus* extracts reconstituted in distilled water and stored at 4°C. The numbers (0 to 52) indicate the duration (week) of storage after reconstitution. Arrow indicates the location of Der p 2.

Protein concentration was determined by Bradford assay, which detects proteins larger than 3 kDa. Glycerol played a protective role against protein degradation, though a decrease in protein concentration was observed even at -20°C. Protein aggregation may have resulted in a decrease in the solubility of proteins, and thus protein concentration, at -20°C when both 0.03% HSA and 50% glycerol were added due to high concentration and low temperature. Furthermore, multiple freezing and thawing cycles may have decreased the allergen potency of the extracts due to protein degradation. More rapid degradation of HSA compared to mite protein may partially explain the more rapid decrease in protein concentration when HSA was added. With regards to Der p 1 concentration, HSA was shown to have a minimal preservative role. Additionally, the overall allergen potency of the extracts was shown to be preserved when glycerol was added at RT.

Soldatova et al.[12] previously reported that competition ELISA is not sufficiently sensitive for detecting decreases in the concentrations of individual allergens. Since epitopes could be recognized by monoclonal or polyclonal antibodies even after partial degradation of individual allergens, they were better able to assess the loss of specific allergens using immunoblotting. That study reported a loss of allergenicity at 6 and 12 months. The addition of protease inhibitors was thought to be unlikely to improve the stability of the mite extracts at 4°C. Liu and Lin, on the other hand, showed no detectable differences in relative potency between extracts kept at 4°C and RT for upto 30 months.[13] However, they compared allergen stability with reference materials stored at 4°C, which also could have undergone degradation. The present data showed that almost 40% of total protein was degraded even though more than 90% of IgE reactivity was preserved during the first two months of storage when reconstituted in 50% glycerol. About 80% of Der p 1 content was found to be preserved in the extracts after two months with the addition of 50% glycerol. These data clearly show that inhibition ELISA is less sensitive to the degradation of individual allergenic components, as some of the partially degraded allergens may retain IgE reactivity. A slow decrease in IgE reactivity compared to the more rapid protein degradation may imply that allergens are structurally stable proteins compared to non-allergenic proteins.

IgE immunoblotting showed that a 15 kDa protein, likely a putative Der p 2, was the most potent allergen in the extracts. This protein exhibited slow degradation over time. A second allergenic component approximately 42 kDa in size was shown to be less stable, though its molecular identity is not yet known. Der p 1 is not easily detected by IgE immunoblotting since its IgE epitope is conformational. Thus, Der p 2 content was not

determined in this study since quantification is unreliable due to its amino acid polymorphism.[14,15] Western blot analysis using antibodies against Der p 1 and Der p 2, and the unidentified 42 kDa allergen may be helpful in future studies to further characterize the stability of HDM extracts.

It may be necessary to investigate the stability of mite extracts in the presence of various protease inhibitors in order to maximize shelf-life. Addition of protease inhibitors has been shown to increase the shelf-life of protease-rich cockroach extracts.[16] HDM is known to contain less protease,[4] however, and the structural integrity of the protein allergen itself remains an important aspect of stability.

This study confirms that storage in refrigerated conditions is the most important factor in maintaining the allergenicity of HDM extracts. Addition of HSA and glycerol may increase the shelf-life of the extracts at RT, though repeated warming for use a RT can result in significant degradation.

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