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Factors influencing the quality of *Myrmecia pilosula* (Jack Jumper) ant venom for use in *in vitro* and *in vivo* diagnosis of allergen sensitization and in allergen immunotherapy

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Short title: Quality of Pharmaceutical grade insect venom extracts for allergen immunotherapy

Summary

Background Allergen immunotherapy uses pharmaceutical preparations derived from naturally occurring source materials, which contain water-soluble allergenic components responsible for allergic reactions. The success of in vivo and in vitro diagnosis in allergen sensitization and allergen immunotherapy largely depends on the quality, composition and uniformity of allergenic materials used to produce the active ingredients, and the formulation employed to prepare finished products.

Objectives We aimed to examine the factors influencing batch-to-batch consistency of Jack Jumper (*Myrmecia pilosula*) ant venom (JJAV) in the form of Active Pharmaceutical Ingredient (AI) and informed whether factors such as temperature, artificial light and container materials influence the quality of JJAV AIs. We also aimed to establish handling and storage requirements of JJAV AIs to ensure preservation of allergenic activities during usage in the diagnosis of allergen sensitization and in allergen immunotherapy.

Methods The quality and consistency of JJAV AIs were analysed using a combination of bicinchoninic acid assay for total protein quantification, HPLC-UV for JJAV allergen peptides quantification, ELISA Inhibition for total allergenic potency, SDS-PAGE, AU-PAGE and Immunoblot for qualitative assessment of JJAV components, and Limulus Amebocyte Lysate assay for the quantification of endotoxin concentration. API-ZYM and Zymogram assays were used to probe the presence of enzymatic activities in JJAV.

Results Pharmaceutical grade JJAV for allergen immunotherapy has good batch-to-batch consistency. Temporary storage at 4°C and light exposure does not affect the quality of JJAV. Exposure to temperature above 40°C degrades high MW allergens in JJAV. Vials containing JJAV must be stored frozen and in upright position during long-term storage.

Conclusions and Clinical relevance We have identified factors which can influence the quality and consistency of JJAV AIs and provided a framework for appropriate handling,

transporting and storage of JJAV to be used for the diagnosis of allergen sensitization and in AIT.

Keywords: Active Pharmaceutical Ingredient, allergen extract, allergen immunotherapy, Jack Jumper ant venom, *Myrmecia pilosula*, pharmaceutical quality

Introduction

Allergy to native ants of the genus *Myrmecia* is an important clinical problem in the south-eastern states of Australia, where Jack Jumper ants (*Myrmecia pilosula*; JJA) are responsible for insect-sting allergy in up to 3% of the population (1, 2). These ants are most prevalent in Tasmania and are the leading cause of anaphylaxis, exceeding reactions caused by bees, wasps, drugs or food (3). Allergen immunotherapy (AIT) against JJA venom (JJAV) is available as an Active Pharmaceutical Ingredient (AI) which is manufactured under current Good Manufacturing Practice (cGMP), and the effectiveness and safety of this treatment has been demonstrated in two published (4, 5) and one recently completed randomized controlled clinical trials (Australian New Zealand Clinical Trials Registry ID: ACTRN12606000333516).

A prerequisite for safe and successful AIT is good quality and uniformity of the administered allergen extracts with a clearly defined composition. When used for diagnostic purposes, allergen extracts must support safe, reliable, and reproducible allergy testing. The composition of JJAV is relatively simple with five predominant low molecular weight (MW) peptides making up approximately 90% of its constituents. Three allergenic peptides (Myr p 1, Myr p 2 and Myr p 3) have been well defined (6). Myr p 2 is the only major allergen in JJAV, whilst Myr p 1 and Myr p 3 are classified as minor allergens (6), and several additional higher MW allergenic components are so far uncharacterised (2). The quantification of these allergenic peptides is therefore relevant for quality assessment and standardization of JJAV preparations.

Standardization and assessment of product quality and stability are essential prerequisites for the manufacture of allergen extracts (7, 8). The reliability of the source material, the extraction process and quality control by the manufacturer significantly influence product quality. Established procedures for allergen standardization must be able to measure potency,

ensure consistency in the composition, and demonstrate the stability of allergen extracts. Using complementary analytical methods, a procedure to standardize JJAV for use in AIT has been developed (9). The procedure is based on the requirements described in the European Pharmacopoeia (EP) Monograph for Allergenic Extracts (10). The ability of these analytical methods to determine stability has been demonstrated previously (11).

The effectiveness of AIT for the treatment of certain insect venom hypersensitivity is reported to be <85% (12, 13) and one possible reason for this is variability in the quality of AI used in the manufacture of insect venom for AIT. Additionally, some patients may be predominantly hypersensitive to “minor” allergens that are not monitored in the manufacturing of an allergenic product and therefore neither presence nor quantities of those allergens are assured, and consequently such patients may not respond to the specific AIT or their response to the AIT may be unpredictable and more liable to vary over time. The aim of this study was therefore to investigate the batch-to-batch consistency of JJAV AI batches produced from different lots of source materials, if factors such as storage temperature, artificial light, container closure materials influence the quality of JJAV AIs post manufacture and to establish handling and storage requirements of JJAV AIs to ensure preservation of allergenic activities during transport and usage.

Materials and Methods

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. All chemicals, reagents and consumables were either analytical, pharmacopoeial, or molecular biology grade.

Jack Jumper ants and the manufacture of active pharmaceutical ingredient grade products

Jack Jumper ants (JJA) were collected from various locations around Tasmania (Australia) and snap frozen at -80°C within 4 hours of collection. Venom sacs, isolated from the abdomen of JJA as previously described (9), were stored at -80°C until further processing. Manufacture of JJAV AI grade products (Fig 1) was performed at the Pharmacy Department, Royal Hobart Hospital (an Australian Therapeutic Goods Administration approved facility to manufacture JJAV AI) according to the cGMP principles (14). Finished products were stored at -80°C until used.

Reference standards

In House Reference Venom

In House Reference (IHR) JJAV was characterized and standardized in early 2000 (15) and was used in a clinical trial, which proved the efficacy of JJA venom immunotherapy (JJA VIT) (4, 9). Aqueous solution of JJAV IHR standard was aliquoted and stored at -80°C until used for the standardization of each JJAV AI batch. Physicochemical stability and allergenic activity of JJAV IHR was continually monitored during quality control.

Serum reference

Pooled Positive Sera (PPS) were prepared from sera of patients with clinically proven history of allergy to JJAV and detection of JJAV-specific IgE-mediated sensitization by skin prick test (SPT) and/or ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden). Blood used in positive serum pools was collected prior to initiation of JJA VIT. Serum was isolated post centrifugation and aliquots were stored at -80°C . Sera from 30 to 40 patients were combined to produce large batches of PPS. The use of patient sera in this study was approved by the Human Research Ethics Committee (Tasmanian) Network and the ethics approval number is H0008934. Informed consent was obtained using written documentation.

Physicochemical analysis

Protein analysis

Total protein concentration in JJAV AI was determined using a bicinchoninic acid (BCA) protein quantification kit (Thermo Fisher Scientific, Rockford, IL) as previously validated (9).

Reverse phase liquid chromatography

High Performance Liquid Chromatography with UV detection (HPLC-UV) was used to quantify the relative amount of Myr p 1 (as Myr p 1.0102 isoform), Myr p 2, and Myr p 3 as previously described (9) with minor modifications. Briefly, an Acquity Ultra Performance Liquid Chromatography system (Waters Corporation, Milford, MA) and Zorbax 300SB-C3, 5 μm , 2.1 x 150 mm reverse phase column (Agilent, Santa Clara, CA) were used with a mobile phase flow rate of 0.3 mL/min which began with acetonitrile:1% trifluoroacetic acid:water (10:8:82), and increased in a linear gradient over 30 min to (70:8:22). Eluents were detected at A_{220} using an Acquity photodiode array detector (Waters Corporation).

JJAV AI batches were analyzed in triplicate by injecting 7.5 µg of venom protein onto the column. Standard curves for Myr p 1, Myr p 2, and Myr p 3 using JJAV IHR contained between 2 to 15 µg of total venom protein per injection and were used to quantify Myr p 1, Myr p 2, and Myr p 3 (TargetLynx Mass Spectrometry software, Waters Corporation). Peptide quantification in venom samples was performed by linear regression.

Electrophoresis and Immunoblotting

For electrophoresis and immunoblotting, JJAV was diluted with Milli-Q water (Milli-Q Biocel, Merck Millipore, Darmstadt, Germany) to 1 mg/mL. Precision Plus Protein WesternC Standards (Bio-Rad, Hercules, CA) was used as molecular weight reference standard. Resolved venom proteins were visualised in-gel by either Coomassie R250 dye (Imperial Protein Stain, Thermo Fisher Scientific) or silver stain (16) or transferred to PVDF membrane (Immobilon P, 0.45 µm pore size, Merck Millipore) for immunoblotting. Stained gels were scanned using a WIA CanoScan 8800F flatbed scanner (Canon Inc., Tokyo, Japan) or Amersham Imager 600 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). For visualisation of immunoblots, Amersham ECL Select Western Blot detection reagent (GE Healthcare) was used. Luminescence was detected using Chemi-Smart 5000 Chemiluminescence imager (Viber Lourmat, Marne la Vallée, France). All image analysis was performed using ImageJ version 1.46r software (Wayne Rasband, National Institutes of Health, Bethesda, MD) (17).

SDS-PAGE

To assess protein composition, degradation and impurities in JJAV AI batches, venom samples were subjected to SDS-PAGE as previously described with minor modifications (9). Venom was heated at 95°C for 5 min in an equal volume of 2x sample buffer prior to electrophoresis using Novex 10–20% Tris-Glycine gels (Life Technologies, Carlsbad, CA). A total of 10 µg venom protein was loaded per lane, except for immunoblot assays where 4 µg of venom protein was added. Gels were electrophoresed at a constant voltage of 125 V for 105 min.

SDS-PAGE Immunoblot

SDS-PAGE Immunoblot was employed to analyse IgE-binding components in JJAV AI batches using a validated method (6, 9) with some modifications. Separated proteins were electrophoretically transferred onto PVDF membrane via a wet transfer method using a TE77

Mighty Small Transphor unit (Amersham Biosciences Corp., Piscataway, NJ) (200 mA for 60 min at 4°C). Subsequently, the membrane was heated in a microwave oven (NEC, Australia) for 5 min at 100% power (850 W) in 400 mL Milli-Q water. Post-heating, membranes were blocked with 1% (w/v) skim milk in Tris-buffered saline (0.02 M; pH 7.4) containing 0.05% polysorbate 20 (TBS-T) for 60 min at room temperature (RT). The membrane was incubated overnight with 1:10 (v/v) dilution of PPS (or negative controls that were serum from individuals non-allergic to JJAV) in antibody dilution solution at RT (Amersham ECL Prime Blocking agent, GE Healthcare). The following morning, the membrane was washed seven times with TBS-T and probed with biotinylated mouse monoclonal anti-human IgE antibody (10 mL of 1:5000 (v/v) dilution in TBS-T of clone HP6029 from either Life Technologies or SouthernBiotech, Birmingham, AL) for 60 min at RT. The membrane was washed seven times with TBS-T and incubated with 10 mL of 1:10,000 (v/v) dilution of streptavidin–horseradish peroxidase conjugate (Life Technologies) in TBS-T for 10 min at RT. After washing the membrane seven times with TBS-T, specific IgE-binding to venom components was detected by chemiluminescence.

Acid Urea PAGE

Acid Urea (AU)-PAGE was used to resolve the 10–17 kDa band in SDS-PAGE gel that contain Myr p 1-3 peptides, as previously described with modifications (18, 19). Briefly, the separating gel consisted of 15% acryl:bisacrylamide (38:2) (Ambion, Carlsbad, CA), 6 M urea (GE Healthcare), and 5.4% acetic acid. The stacking gel consisted of 7.5% acryl:bisacrylamide (38:2), 6 M urea, 360 mM acetic acid, and 50 mM potassium acetate. Venom samples were mixed with equal volume of sample buffer (6 M urea, 250 mM acetic acid, 100 mM potassium acetate, 15% (w/v) sucrose, and 0.001% (w/v) methyl green) and 5 µg of venom protein/lane were separated at 200 V for 90 min.

AU-PAGE Immunoblot

AU-PAGE immunoblots were used to analyse IgE-binding to the Myr p 1-3 peptide bands. AU-PAGE resolved JJAV peptides were electrophoretically transferred to a PVDF membrane using a TE70 ECL Semidry transfer unit (Amersham Biosciences Corp.) and semi-dry transfer method at 1.5 mA/cm² as previously described (6) and specific IgE-binding to venom components was detected by chemiluminescence.

Allergen analysis

ELISA Inhibition

The allergenic potency of JJAV AI batches was determined using a JJAV-specific IgE ELISA inhibition assay as previously described with some modifications (9). 96-well Amino Immobilizer plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 µL of 10 µg/mL JJAV in sodium carbonate (0.05 M, pH 9.6). Inhibition mixtures were prepared by adding between 0.15 and 4 ng of JJAV to 25 µL PPS and diluting to 100 µL with phosphate buffered saline (0.01 M, pH = 7.2) containing 0.05% polysorbate 20 (PBS-T) and 0.5% bovine serum albumin (BSA); JJAV was either JJAV IHR or JJAV AI batch. A positive control was prepared by diluting PPS 1:4 (v/v) in PBS-T with 0.5% BSA and negative control consisted of PBS-T with 0.5% BSA and incubated overnight at 4°C. Subsequently, venom coating solution was discarded and the plate was washed three times with PBS-T. The venom-coated plate was incubated with 100 µL of inhibition mixtures, positive control and negative control for 60 min at RT. The plate was washed three times with PBS-T, and 100 µL of biotinylated mouse monoclonal anti-human IgE antibody (1:2000 (v/v) dilution in PBS-T, clone HP6029) was added to each well and incubated in the dark for 60 min at RT. The plate was washed three times with PBS-T, and then 100 µL of streptavidin–horseradish peroxidase (1:1000 (v/v) dilution in PBS-T, BD Pharmingen, San Diego, CA) was added to each well and the plate was incubated in the dark for 20 min at RT. The plate was washed three times with PBS-T, and then 100 µL of 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific) was added to each well and the plate was incubated in the dark for 30 min at RT. Colour development was stopped by adding 100 µL of 2 M sulphuric acid and absorbance was read at 450 nm utilizing an ELISA plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA). Samples were run in three independently prepared replicates, and the mean, standard deviation, and coefficient of variation calculated. The percentage inhibition for each sample was calculated using the following equation:

$$\text{Inhibition of IgE binding (\%)} = \frac{\text{Absorption of positive control} - \text{Absorption of inhibited sample}}{\text{Absorption of positive control}}$$

Allergenic potency of JJAV was measured as the amount of venom in solution that was required to inhibit 50% (ID₅₀) of IgE binding to the JJAV coated onto the well.

Biological analysis

Endotoxin content

Endotoxin levels were determined using Limulus Amebocyte Lysate (LAL) QCL-1000 chromogenic assay (Lonza, Walkersville, MD) according to the manufacturer's instructions. Colour development was stopped by the addition of 100 μ L of 25% (v/v) acetic acid and the absorbance was measured at 405 nm using a plate reader (SpectraMax M2, Molecular Devices). Endotoxin levels in JJAV samples were calculated from a standard curve containing between 0 to 1 EU/mL *E. coli* 0111:B4 Endotoxin (Lonza).

Enzymatic activities

JJAV AI was diluted to a concentration of 1 mg/mL in Eppendorf Biopur microcentrifuge tubes (Eppendorf AG) using Water for Injection B.P. (Pfizer, West Ryde, NSW, Australia).

API-ZYM system

Semi-quantitative analysis of enzyme content of JJAV was performed using the API-ZYM system (BioMérieux, Marcy-l'Étoile, France) to measure the activity of 19 different types of enzymes, including 5 proteases, 3 lipases, 3 phosphatases, and 8 glycosidases. The assay was performed according to the manufacturer's instructions using 65 μ g venom protein/well. The approximate amount of product formed was semi-quantitatively assessed according to the API-ZYM reading colour-scale (20-22).

Zymogram assay

The presence of matrix metalloproteinases in JJAV was determined using Novex Zymogram 4–16% Tris-Glycine gels containing 0.1% blue-stained β -casein as the substrate (Life Technologies) according to the manufacturer's instructions.

Batch-to-batch consistency

To study the batch-to-batch consistency, all batches of JJAV AI manufactured between 2008 and 2015 were included in the analysis. All samples were analysed using the validated BCA protein quantitation, ELISA Inhibition, HPLC-UV, SDS-PAGE, SDS-PAGE Immunoblot and Endotoxin assay methods. Results were analysed relative to JJAV IHR, which was included in each analysis, except in the endotoxin assay where JJAV IHR was omitted. The mean, standard deviation, and coefficient of variation were calculated for quantitative assays.

Studies on storage requirements

For stability studies of JJAV AI, venom concentration was adjusted to 1 mg/mL with Water for Injection B.P. in a sterilized Protein LoBind microcentrifuge tube (Eppendorf AG). Venom aliquots were transferred into sterile USP Type 1 clear glass borosilicate vials and sealed with sterile grey butyl rubber stoppers (both from Wheaton, Millville, NJ). Venom, immediately transferred to -80°C post preparation served as baseline.

Studies were conducted using 2 independently prepared samples from different batches of JJAV AI. Treatment, control and baseline samples were analysed using ELISA Inhibition, HPLC-UV, SDS-PAGE, AU-PAGE, SDS-PAGE Immunoblot and AU-PAGE Immunoblot methodologies.

Adsorption of JJAV allergenic components to rubber stopper

Treatment samples were stored inverted (to allow full contact of venom solution with the rubber stopper) and control samples were stored upright; all samples were protected from light at 4°C for up to 48 hours. Treatment and control samples were collected at 6, 24, and 48 hours, and were stored at -80°C until analysis.

Effect of light and storage temperature on JJAV allergenic components

Treatment samples were stored upright for up to 48 hours at either 4°C or 25°C and continuously exposed to artificial light (white fluorescent light with illuminance intensity between 2200 and 2500 lux, with samples positioned 30 to 60 cm from the light source) throughout the storage period. Control samples were aliquots of the same venom preparation that were treated identically other than being protected from light. Temperature and humidity of the storage areas were monitored using a temperature data logger (EasyLog, Lascar Electronics, Salisbury, UK). The mean and SD of temperatures and humidity in the storage area during the study period were controlled at $4.6 \pm 0.4^{\circ}\text{C}$ and $24.9 \pm 0.5^{\circ}\text{C}$, and $76.7 \pm 5.2\%$ RH and $37.1 \pm 7.7\%$ RH, respectively. Treatment and control samples were collected after 24 and 48 hours of incubation and were stored at -80°C until analysis.

Effect of elevated temperature on JJAV allergenic components

Venom samples were incubated at 20°C , 40°C , 60°C , or 80°C for 2 hours in the dark, after which they were stored at -80°C until analysis.

Data analysis

All statistical analysis was performed using GraphPad Prism 5.03 and CurveExpert Basic 1.4. Statistical analyses, including descriptive statistics, ANOVA with Dunnett's post-hoc test, and Spearman correlation analysis were used as appropriate. Calculated p-values were two-tailed unless otherwise stated; $p < 0.05$ was considered significant.

Acceptance Criteria

The following acceptance criteria (based on the requirements set in the European Pharmacopoeia (EP) Monograph for Allergen Products (10)), were used for standardizing JJAV AIs:

- Venom protein concentration: adjusted to between 80 and 120% of JJAV IHR
- ELISA Inhibition: the 50% inhibition value is between 50 and 150% of JJAV IHR
- HPLC-UV: the amount of Myr p 1, Myr p 2, and Myr p 3 are between 50 and 200% of JJAV IHR
- SDS-PAGE: presence of bands at approximately 10-17, 22, 27, 29, 33, 45, 70, 90 and 250 kDa
- SDS-PAGE Immunoblot: presence of prominent IgE-binding bands at approximately 10-17 and 22-23 kDa.

Results

JJAV AI batch-to-batch consistency

Out of 17 batches of JJAV AI, 3779 ± 917 (mean $\pm$ standard deviation (SD)) venom sacs were used (Fig 2). The total amount of venom protein in JJAV AI batches was 134 ± 24 mg (Fig 2). The relative allergenic potency for JJAV AI batches was $80.9 \pm 17.4\%$ compared to IHR JJAV (Fig 3). HPLC-UV analysis of JJAV AIs showed very similar chromatograms comparable to the JJAV IHR (see Fig S1 for a typical chromatogram of JJAV IHR). Characteristic peaks corresponding to the Myr p 1-3 peptides were detected at identical retention times with similar peak areas to the JJAV IHR for the different batches of JJAV AIs with no apparent impurities or degradation peaks. The mean and SD calculated amounts of Myr p 1, Myr p 2 and Myr p 3 allergen peptides in JJAV AI batches were $96.7 \pm 10.4\%$, $99.5 \pm 9.2\%$, and $93.2 \pm 8.9\%$ relative to the JJAV IHR, respectively (Fig 3). All JJAV AI batches were well within the limits of the pre-defined acceptance criteria. JJAV contained very low

amounts of endotoxin. The mean and SD of calculated endotoxin concentrations in JJAV AIs was 0.039 ± 0.0122 EU/mL (Fig 4).

There were strong correlations ($r > 0.77$, $p = 0.0003$ or less) between all of the quantified allergen peptides (Fig 5, A – C). Moderate correlations were identified for the relative total allergenic potency of JJAV AI *versus* relative amount of Myr p 3 ($r = -0.4224$, $p = 0.0456$) (Fig 5, D) and for the relative total allergenic potency of JJAV AI *versus* endotoxin concentration ($r = 0.5445$, $p = 0.0238$) (Fig 5, E). No correlation was found with other parameters analysed.

The apparent molecular weight of proteins and peptides in JJAV range from approximately 6.6 to 250 kDa and the most prominent bands are in the region of 10-17 kDa. No major changes in JJAV protein composition (harvested and pooled) within the study period were detected since identical band profiles and similar staining intensities between different batches of JJAV AI were observed (Fig S2, A). Up to eleven distinct IgE-binding venom bands were detected by SDS-PAGE Immunoblot, with the main allergenic band in JJAV found between 10-17 kDa (Fig S2, B). Comparable allergen patterns and similar consistency of luminescence intensity of IgE-binding bands were found between different batches of JJAV AI and JJAV IHR.

Non-heated JJAV AI possessed 8 types of strong enzymatic activities (i.e. >30 nanomoles of hydrolyzed substrate/65 μ g venom protein), and 4 types of weak (or negative) enzymatic activities (i.e. <10 nanomoles of hydrolyzed substrate/65 μ g venom protein) (Table 1). The most activity was observed for phosphatases, while moderate leucine aminopeptidase activity was present. No matrix metalloproteinase activity was observed in any JJAV AI batches (data not shown). After 2 hours, JJAV heated to 60°C resulted in significantly decreased enzymatic activities while no enzymatic activities remained after heat treatment at 80°C. The heat-reduced enzymatic activity pattern in venom samples closely mirrored the protein degradation pattern derived from SDS-PAGE analysis (Fig S3, A).

Effect of rubber stoppers on JJAV components and potency

HPLC-UV analysis of JJAV AI indicated that peptide loss occurred only after the venom solution was in contact with rubber stopper for at least 24 hours. The largest venom peptide loss was observed for Myr p 1 with up to 14.4% reduction after 48 hours relative to the baseline (Fig 6, A). This venom exhibited slightly stronger allergenic potency compared to venom samples with shorter duration of contact, baseline sample, or JJAV IHR (Fig 7, C). Image analysis from both SDS- and AU-PAGE did not show qualitative changes with regards to band size or intensity (Fig S4, A and C). No reduction in the intensity of any of the allergenic bands was observed from the immunoblot assay results (Fig S4, B and D).

Effect of storage temperature and artificial light on the stability of JJAV

When compared with the baseline sample, HPLC-UV data showed no statistically significant loss in any quantified peptides when JJAV was incubated at 4°C for up to 48 hours (Fig 6, B). In contrast, a trend towards decreased amounts of peptides, particularly Myr p 3, was observed in venom samples incubated at 25°C for 48 hours, although the reduction did not reach statistical significance (Fig 6, C). No differences were observed between samples exposed to, or protected from artificial light for the period studied (Fig 6, B and C).

SDS-PAGE and SDS-PAGE Immunoblot analysis revealed some loss of higher MW components in JJAV samples incubated at 25°C for 48 hours (Fig S5, A and B). Storage of JJAV AI at RT was found to affect the stability of the 22-23 kDa IgE-binding duplet bands, with degradation of the 23 kDa band clearly evident at 48 hours duration regardless of light exposure. The degradation was accompanied by the development of a new IgE-binding band at 21 kDa. Additionally, when compared to baseline sample, a loss of an IgE-binding band at approximately 124 kDa was also evident in all venom samples incubated at 25°C. In comparison, no degradation of epitopes, indicated by unchanged luminescence intensity and/or development of new IgE-binding bands was detected in any of the JJAV samples stored at 4°C. Image analysis from both AU-PAGE and AU-PAGE Immunoblot assays did not detect qualitative changes to the allergenic peptide band size or intensity for all conditions tested (Fig S5, C and D).

Allergenic potency, as determined by ELISA Inhibition, was unchanged in venom samples stored at 4°C, whilst allergenic potency increased in venom samples stored at 25°C (Fig 7, A and B).

Effect of elevated temperature on stability of higher MW components in JJAV

JJAV AI was found to be thermally stable for up to 2 hours at 40°C, but there was precipitate formation when heated to 60°C and 80°C. HPLC-UV analysis of venom stored at 80°C for 2 hours demonstrated a 16.9% and 12.8% reduction in the level of Myr p 1 ($p < 0.01$) and Myr p 2 ($p < 0.05$) relative to the baseline sample (Fig 6, D). In contrast, the amount of Myr p 3 appeared to increase as the incubation temperature increased, with JJAV heated to 80°C demonstrating a 22.2% increase relative to the baseline sample ($p < 0.001$). ELISA Inhibition indicated increased allergenic potency as venom was heated from 20°C to 80°C, with allergenic potency of the venom sample heated to 80°C was found to be 1.5 and 2-fold higher than the baseline ($p > 0.05$) and JJAV IHR ($p < 0.05$), respectively (Fig 7, D).

SDS- and AU-PAGE experiments showed that the high MW venom components were temperature sensitive and appeared to fragment to smaller components when heated to 60°C and 80°C, while the lower MW venom components remained relatively unaffected (Fig S3, A and C). Immunoblotting experiments were consistent with this observation. Degradation of the epitopes within the 22-23 kDa IgE-binding bands was evident from 60°C and the epitopes were completely lost after storage at 80°C. This loss was accompanied by the development of a new IgE-binding band below the 10 kDa region in the SDS-PAGE Immunoblot (Fig S3, B). In contrast, no loss in IgE-binding capability of the Myr p allergens was observed in AU-PAGE Immunoblot assay (Fig S3, D).

Discussion

The primary finding of this study is that JJAV AIs have good batch-to-batch consistency, which may be an important reason why JJA VIT has a real-world efficacy of >95% (23), and it remains to be seen if other *hymenoptera* venom AI for AIT has similar batch-to-batch consistency. Relative amounts of allergenic peptides Myr p 1-3 are strongly correlated to each other, and the relative amounts of Myr p 3 and the levels of endotoxin concentration were found to correlate with total allergenic potency of the product. Several external factors that affect the quality of JJAV AIs have been identified. Prolonged storage at or above RT or contact with rubber stoppers is detrimental to the quality and consistency of JJAV AIs.

The strength of this study is the number of samples included in the analysis; the study included 17 batches of JJAV AIs spanning 7 production years. In addition, in the storage requirement studies, duplicates of samples from different batches of JJAV AIs were independently prepared, treated and analysed. As only single batch analysis is typically

required when conducting stress studies (24), the results generated from this study are particularly robust. However, we have not yet identified the higher MW allergens and were not able to quantify these allergens, although this is not required according to EP Monograph.

Natural allergen extracts used in the diagnosis of allergen sensitization and in AIT are complex mixtures of biological materials. Major limitations of natural allergen extracts are the potential for variable composition, allergen content and the difficulties of standardization of allergenic contents (25). Poor quality of allergenic products has the potential to affect treatment outcomes in patients that require VIT. It is therefore imperative that JJAV AIs must show good batch-to-batch consistency within strict specifications as defined in the relevant Pharmacopoeia.

Seasonal variability has been noted for allergens from other species (26, 27), which is minimised with JJAV by pooling venom from ants collected at different times and from different locations. Using this strategy, our analysis revealed good batch-to-batch consistency and homogeneity. The total protein concentration and relative allergenic potency of JJAV AIs showed < 2-fold variation between batches and the amounts of allergenic peptides Myr p 1-3 were 77 to 118% of the content in JJAV IHR. The higher MW proteins and allergenic profile were also found to be similar between batches and comparable to the JJAV IHR profile.

Endotoxins derived from the cell walls of Gram-negative bacteria are potent pro-inflammatory compounds that have been shown to cause both acute and chronic illnesses, including fever, organ failure and death. Testing for endotoxin levels is mandatory for parenterally administered substances and endotoxin levels as high as 33,800 EU/mL have been reported in some AIT products (28). JJAV however consistently showed very low endotoxin concentrations below 0.055 EU/mL (equivalent to 0.011 EU/mg of venom protein). Considering that the maximum endotoxin limit in drugs and biological materials is 5 EU/kg/h (equivalent to 8400 EU/day for a 70 kg patient) (28, 29), the endotoxin concentration in JJAV AIs is approximately 8×10^6 -fold lower than the acceptable limit.

In the stress-testing studies, a decrease in the amount of Myr p 1 and to a lesser degree Myr p 3 was observed when JJAV solution was in contact with rubber stoppers for more than 24 hours. Adsorption of allergenic proteins to the surface of glass vials has been reported previously (30) and evidence from stability studies showed that JJAV adsorbs to container surface material at lower venom concentrations (11). This study provides evidence that surface adsorption of JJAV components by closure materials still takes place even at high

venom protein concentrations. Exposure to artificial light and storage at refrigerated temperatures for up to 48 hours had negligible effects on the quality of JJAV AI, whereas JJAV allergens in the AI were unstable at RT. Specifically, reduced concentrations of Myr p 1, Myr p 3, and the 23 kDa and 124 kDa proteins were apparent after as little as 24 hours. Our observation on allergen content in JJAV AI at different temperatures supports previous reports on other types of allergenic products (30, 31). Similar results with the reduction in Myr p peptides and degradation of the 23 kDa allergen, which is recognised by 46.3% of JJAV allergic individuals (6) have been reported in samples containing lower concentration of JJAV (100 µg/mL) formulated in 2% sucrose, phosphate buffered saline (10 mM, pH 6), 0.05% polysorbate 80 and 0.9% benzyl alcohol (11). Our study provides additional evidence for instability of the 124 kDa allergenic protein in JJAV when stored at RT for 24 hours or longer. Degradation of the 23 kDa and 124 kDa allergenic proteins may potentially cause false negative results if the product was used for diagnostic purposes, or treatment failure when used in AIT in those patients who are sensitive to these allergens.

Exposure to different temperatures demonstrates considerable variation in the thermal stability of JJAV components. Based on the HPLC-UV, AU-PAGE and Immunoblot analysis, it was clear that the allergenic Myr p peptides in JJAV and their IgE epitopes are relatively thermostable even after heating for 2 hours at 80°C. In comparison, results from the SDS-PAGE and Immunoblot analysis provides evidence that the high MW proteins in JJAV are readily destroyed at elevated temperatures and are therefore likely to be responsible for the precipitations observed.

Heat exposure of allergenic products is frequently accompanied by a reduction in allergenic activity, allergenic potency, or allergen concentration (32-35). In this study however, a trend for increased allergenic potency and Myr p 3 concentration were observed upon exposure to elevated temperatures. This phenomenon of increased allergenic potency secondary to thermal processing has also been reported in roasted peanuts (36-39). A possible explanation for this is likely to be associated with the aggregation-state of JJAV allergens. Myr p peptides, which contribute to the majority of allergenic activities in JJAV, are amphiphilic in nature and have the potential to form aggregates (11). An overestimation in allergenic potency is likely if IgE epitopes are concentrated, or an underestimation if IgE epitopes are hidden within the aggregates (11, 40-43). The apparent increase in Myr p 3 concentration at elevated temperatures may be due to the formation of a minor degradation product or aggregation of Myr p peptides with retention time similar to the native allergen

(11), which has been reported for other allergens (44, 45); in all cases, measured allergen levels were found to increase upon heating at 80°C or above.

The observation that IgE binding of Myr p peptides remains unaltered despite heating at 80°C for 2 hours allows us to speculate on the nature of IgE epitopes in these allergenic peptides. Heating of protein and peptide allergens is likely to denature their three-dimensional structure, thus reducing the binding affinity of IgE to conformational epitopes. Furthermore, the use of SDS in the SDS-PAGE and Immunoblot analysis is also expected to denature proteins in the process, which may impact on conformational venom epitopes (46). The resistance of the Myr p peptides to elevated temperatures, together with the detection of allergenic activity in the denaturing condition of SDS-PAGE Immunoblot assay, strongly suggests that Myr p peptides contain sequence-specific rather than conformational epitopes.

Enzymatic activities in allergenic extracts, particularly proteases, are known to negatively affect the quality and activity of allergenic products (47-51). JJAV possesses various enzymatic activities (2), however no studies had described the presence of proteases in this venom to date. We detected one type of protease (leucine aminopeptidase) in JJAV with moderate levels of activity, and it is possible that this endogenous protease degrades high MW proteins in JJAV AI (20). Apart from this protease, acid and alkaline phosphatase activities detected in this study are consistent with published data (2), whereas the glycosidase activities observed have not been reported before. Previously, proteases and acid phosphatases in allergenic extracts have been described to correlate with allergenic activities or allergenic potencies (52, 53). In contrast to the Myr p peptides, both protease and phosphatase enzymes in JJAV were highly sensitive to heat treatment and were rapidly lost at temperatures above 55°C.

Conclusion

Based on this study the following guidelines are recommended for storage, handling and transport of JJAV AIs:

- i. Storage of JJAV AIs at very low temperature (-20°C or below) will ensure long term preservation of JJAV allergenic activity (11). If necessary, temporary storage at +4°C for up to 48 hours will not affect the quality of JJAV AIs.
- ii. Exposure to elevated temperatures (above +40°C) degrades high MW allergens in JJAV within 2 hours.

- iii. Short exposure to artificial light (for up to 48 hours) does not affect the quality of JJAV AIs.
- iv. Short exposure of JJAV AIs to rubber stoppers (up to 24 hours) will not affect product quality significantly, but vials containing JJAV AIs must be stored in an upright position during long-term storage to avoid contact for extended periods of time.

By identifying factors which can influence the quality and consistency of JJAV AIs, we have provided a framework for appropriate handling, transporting and storage of JJAV to be used for the diagnosis of allergen sensitization and in AIT.

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Contributors

SGAB and MW developed the venom extraction, pooling and handling procedures relevant to the study; TW conceived and designed the experiments; TW and HED performed the experiments; NWD and DN performed and analysed the HPLC-UV experiments; TW, HED and LMCL analysed the data from JJAV AIs batch analysis. All authors contributed to the writing of this manuscript.

Conflict of interest

TW, HED, LMCL, SGAB, and MDW are either employee or consultants to the Jack Jumper Allergy Program, the organization owning the intellectual property over Jack Jumper Ant Venom Immunotherapy. All other authors declare no conflict of interest.

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Table 1. Effects of elevated temperatures on enzymatic activities of Jack Jumper Ant Venom Active Pharmaceutical Ingredients

Enzymes		JJAV Samples		
		Untreated	Heated to 60°C	Heated to 80°C
Proteases	Leucine aminopeptidase	3	1	0
	Valine aminopeptidase	0	0	0
	Cystine aminopeptidase	0	0	0
	Trypsin	0	0	0
	α -chymotrypsin	0	0	0
Lipases	Esterase	3	0	0
	Esterase lipase	1	0	0
	Lipase	0	0	0
Phosphatases	Alkaline phosphatase	5	0	0
	Acid phosphatase	5	1	0
	Phosphoamidase	4	1	1
Glycosidases	α -galactosidase	1	0	0
	β -galactosidase	2	0	0
	β -glucuronidase	0	0	0
	α -glucosidase	4	0	0
	β -glucosidase	1	0	0
	N-acetyl- β -glucosaminidase	4	1	0
	α -mannosidase	0	0	0
	α -fucosidase	3	2	0

1= 5 nanomoles of hydrolysed substrate/65 μ g venom protein; 2 = 10 nanomoles of hydrolysed substrate/65 μ g venom protein; 3 = 20 nanomoles of hydrolysed substrate/65 μ g venom protein; 4 = 30 nanomoles of hydrolysed substrate/65 μ g venom protein; 5 = >40 nanomoles or more of hydrolysed substrate/65 μ g venom protein. Values above 2 are considered positive for enzymatic activity. JJAV samples were analysed at a concentration of 1 mg/mL.

Figure Legends

Figure 1. Flow scheme for the manufacture of JJAV AI grade products. The scheme summarizes the manufacturing steps involve from raw material collection until the release of JJAV as AIs grade products. B.P., British Pharmacopoeia; QC, Quality Control; QA, Quality Assurance.

Figure 2. Batch-to-batch consistency of JJAV AIs measured in terms of venom sac content and venom protein concentration in the batch. VS: Number of venom sac used in 17 JJAV AI batches, TP: total amount of venom protein in 17 JJAV AI batches as determined by BCA protein quantification assay. Horizontal lines indicate mean and standard deviation.

Figure 3. Batch-to batch consistency of 17 JJAV AI batches measured in terms of individual allergenic peptides concentration and total venom potency relative to JJAV IHR. Myr p 1: quantified amount of Myr p 1 peptide in JJAV AI batch relative to JJAV IHR, Myr p 2: quantified amount of Myr p 2 peptide in JJAV AI batch relative to JJAV IHR, Myr p 3: quantified amount of Myr p 3 peptide in JJAV AI batch relative to JJAV IHR, ID₅₀: calculated 50% inhibition of JJAV AI batch relative to JJAV IHR. Horizontal lines indicate mean and standard deviation.

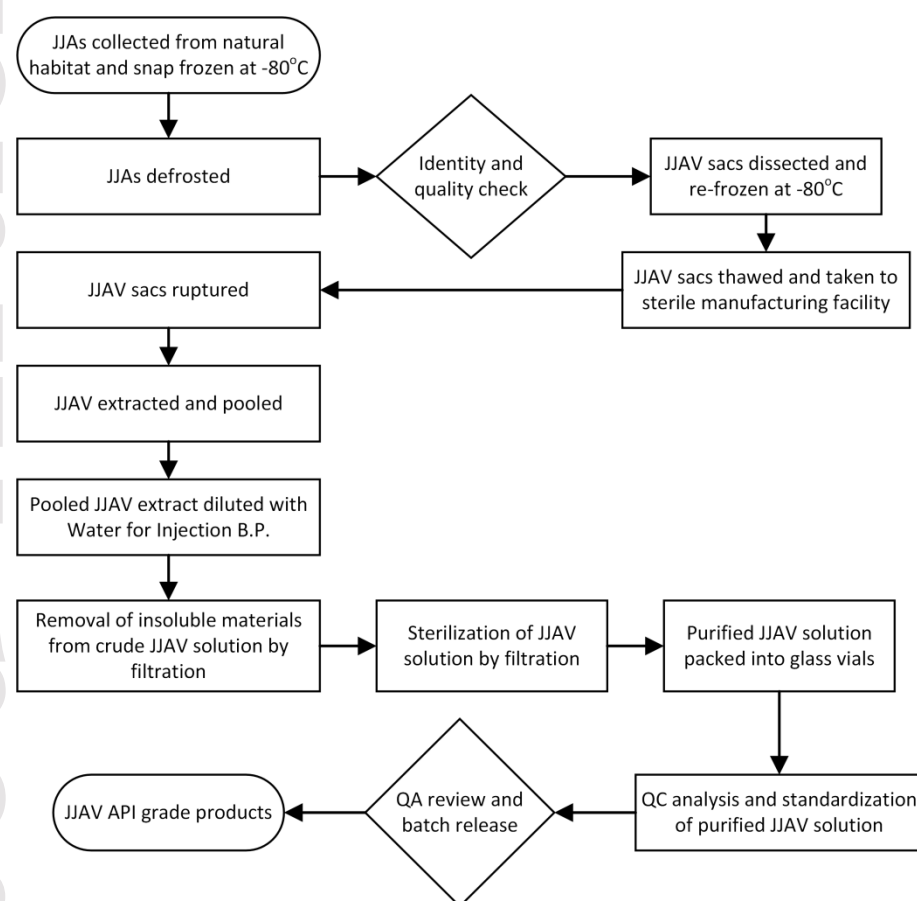
Figure 4. Endotoxin concentration in 17 batches of JJAV AIs. Endotoxin concentration in JJAV samples were measured using LAL QCL-1000 chromogenic assay. Horizontal lines indicate mean and standard deviation.

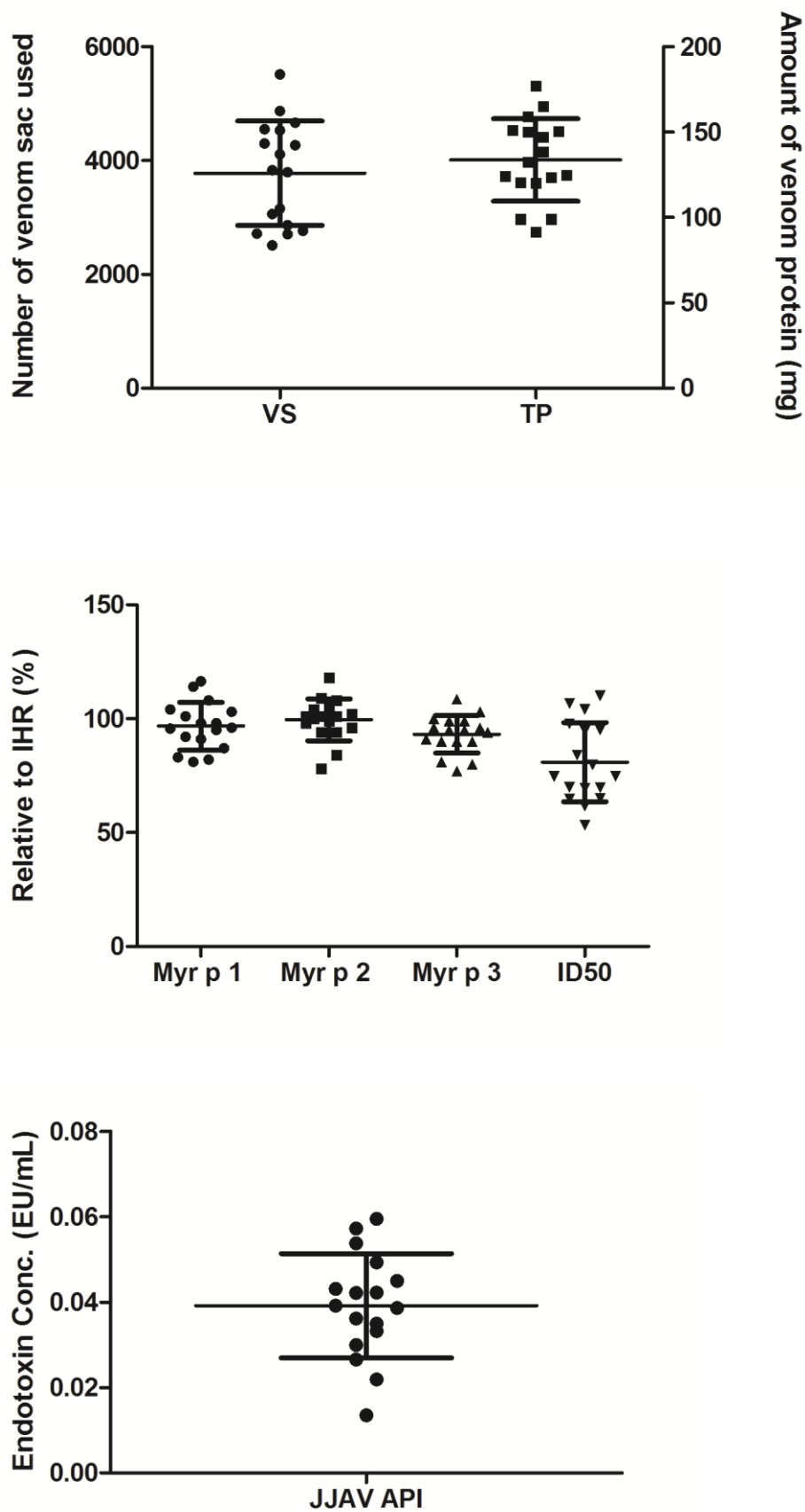
Figure 5. Scatter plots of Spearman correlation analysis generated from the batch-to-batch analyses of JJAV AIs. Figures A – C and E: Parameters fitting Spearman correlation with 2-tailed analysis and 99% confidence interval (CI); Figure D: Parameters fitting Spearman correlation with 1-tailed analysis and 90% CI.

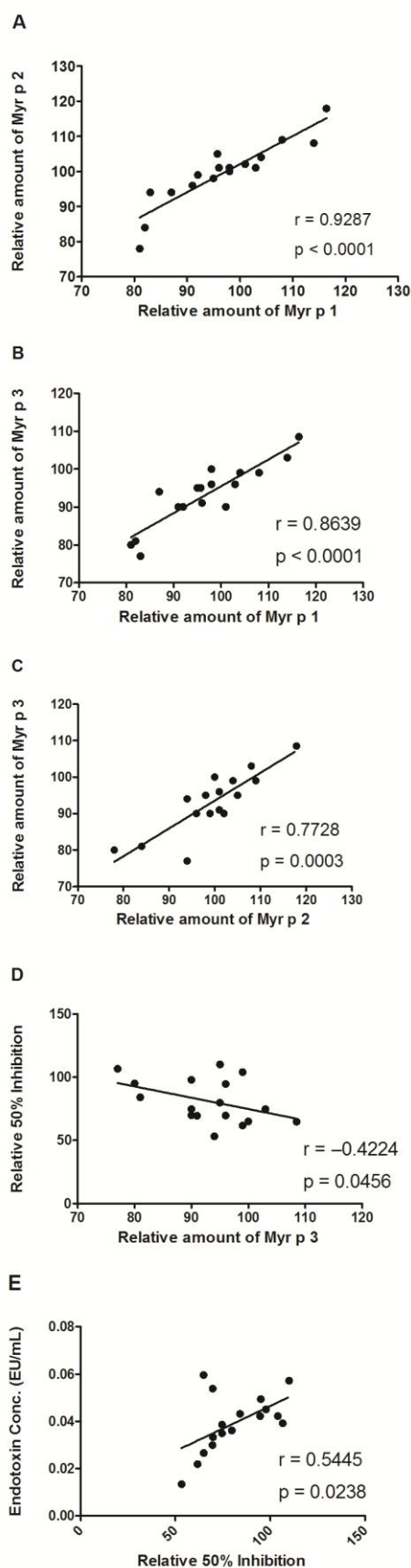
Figure 6. Analysis of JJAV AIs samples showing the effects of different storage conditions on the quantified JJAV peptides Myr p 1, Myr p 2 and Myr p 3. 1 mg/mL JJAV AIs were exposed to rubber stoppers internal surface at 4°C for up to 48 hours (A), or exposed to artificial light at 4°C for up to 48 hours (B), or exposed to artificial light at 25°C for up to 48 hours (C), or exposed to elevated temperatures for 2 hours (D). Samples from different batches of JJAV AIs (n = 2) were independently prepared and HPLC-UV analysis was performed in triplicate. * p < 0.05 compared to baseline venom sample, ** p < 0.01 compared to baseline venom sample, *** p < 0.001 compared to baseline venom sample. Error bars indicate standard error of the mean (SEM).

Figure 7. The effect of different storage conditions on allergenic potency of JJAV. JJAV AIs (1 mg/mL) were exposed to artificial light at 4°C for up to 48 hours (A), or exposed to artificial light at 25°C for up to 48 hours (B), or exposed to rubber stoppers internal surface at 4°C for up to 48 hours (C), or exposed to elevated temperatures for 2 hours (D). Samples from different batches of JJAV AIs (n = 2) were independently prepared and sample analysis by ELISA Inhibition was performed in duplicate. Logistic regression analysis was used to

calculate 50% inhibition of JJAV-specific IgE binding to JJAV coated to ELISA plate. * $p < 0.05$ compared to JJAV IHR. Error bars indicate SEM.

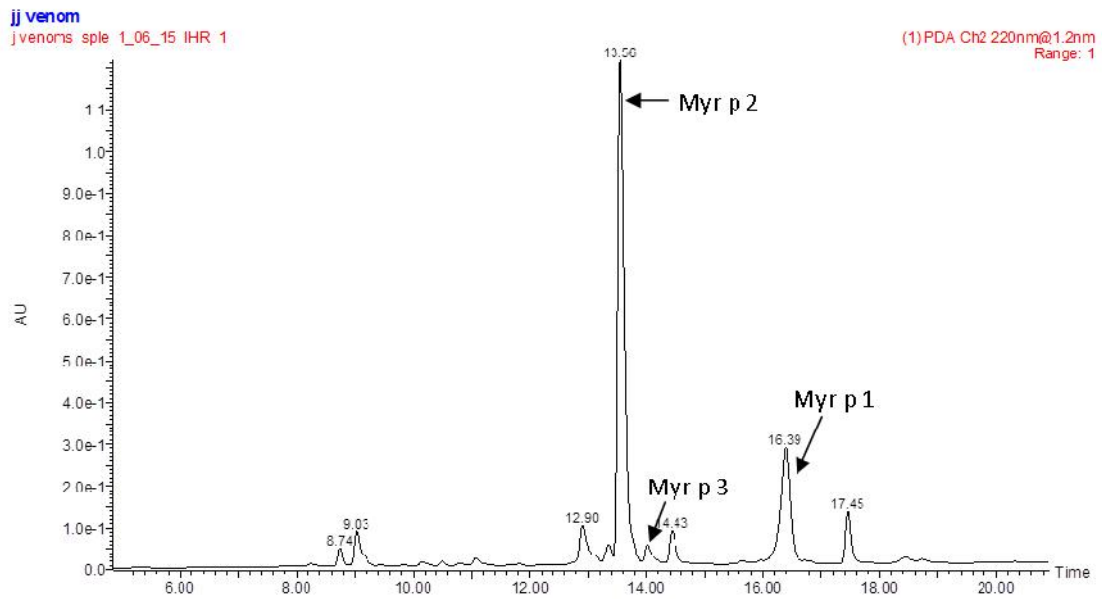






1 Supporting Information

2

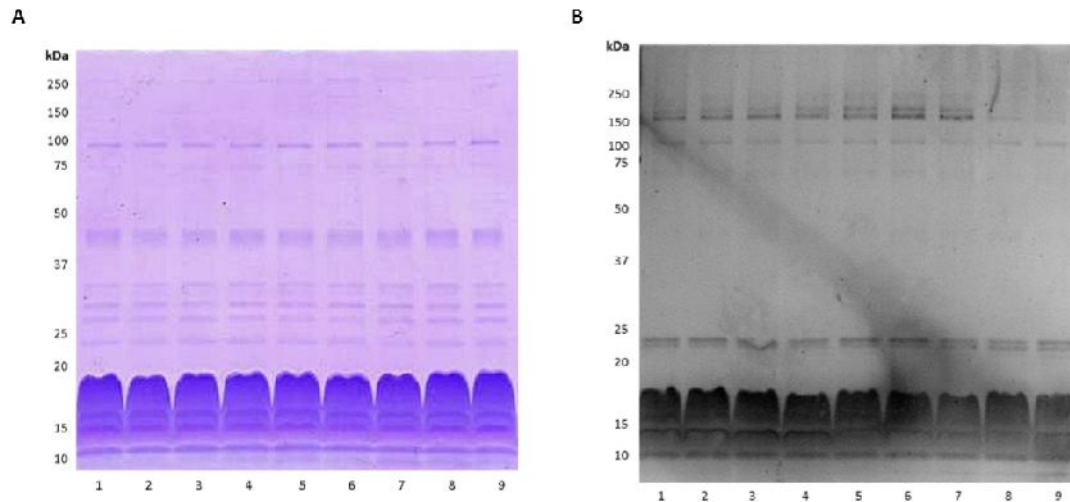


3

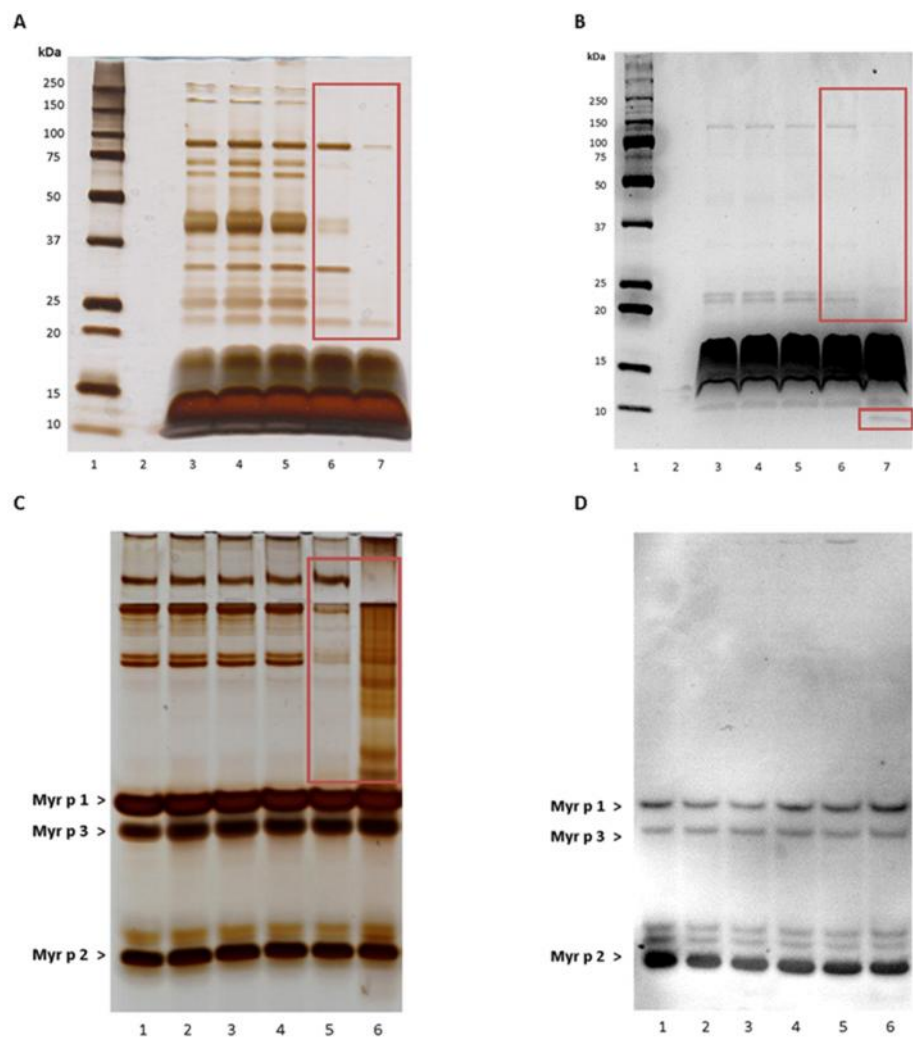
4

5 **Supplemental Figure 1.** A typical HPLC-UV chromatogram of JJAV IHR showing
6 the quantified peaks and retention times corresponding to the allergenic peptides Myr
7 p 1, Myr p 2, and Myr p 3.

8

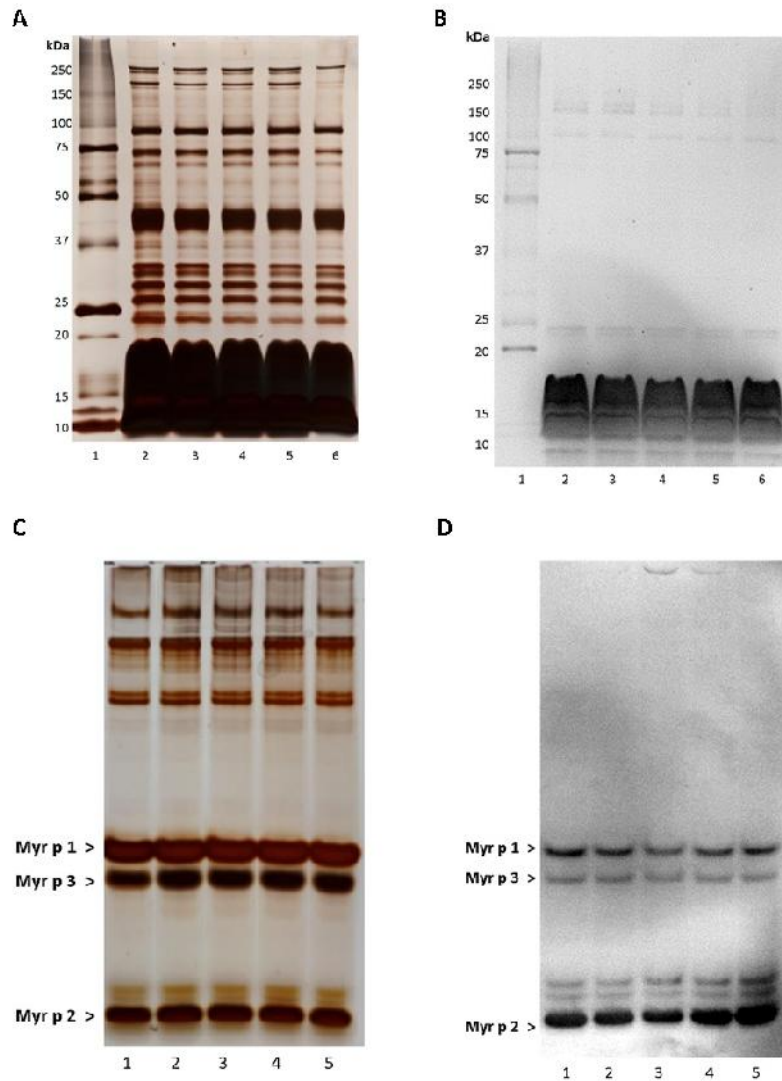


Supplemental Figure 2. Batch-to-batch consistency of JJAV APIs as analysed using SDS-PAGE with Imperial Protein stain (A) and SDS-PAGE Immunoblot (B) methodologies. Lanes: 1. JJAV IHR, 2. API batch 2-02, 3. API batch 2-03, 4. API batch 3-01, 5. API batch 3-02, 6. API batch 3-03, 7. API batch 4-01, 8. API batch 5-01, 9. API batch 5-02.

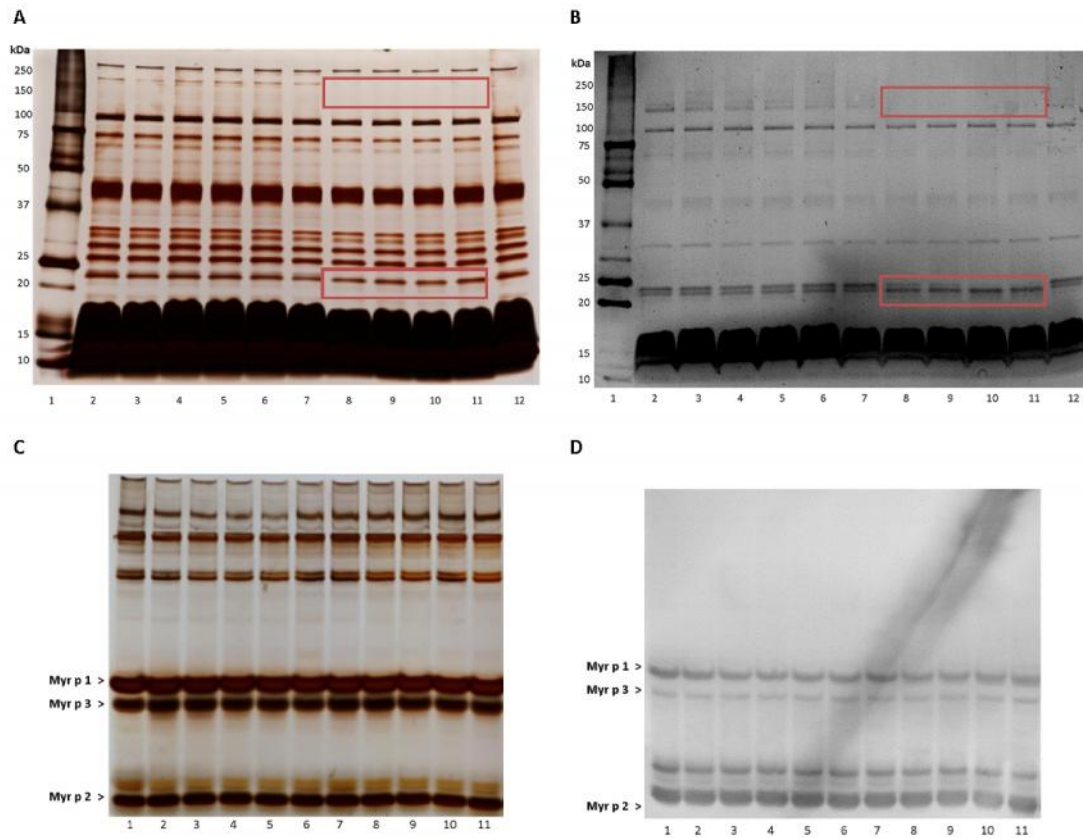


17

18 **Supplemental Figure 3.** Effects of elevated temperature on the stability of JJAV
19 analysed using SDS-PAGE with silver stain (A), SDS-PAGE Immunoblot (B), AU-
20 PAGE with silver stain (C), and AU-PAGE Immunoblot (D) methodologies. JJAV
21 APIs (1 mg/mL) were incubated at various temperatures for 2 hours. For figures (A)
22 and (B) lanes: 1. MW standards, 2. Empty, 3. Baseline sample, 4. Treatment sample
23 incubated at 20°C, 5. Treatment sample incubated at 40°C, 6. Treatment sample
24 incubated at 60°C, 7. Treatment sample incubated at 80°C. For figures (C) and (D)
25 lanes: 1. JJAV IHR, 2. Baseline sample, 3. Treatment sample incubated at 20°C, 4.
26 Treatment sample incubated at 40°C, 5. Treatment sample incubated at 60°C, and 6.
27 Treatment sample incubated at 80°C.



Supplemental Figure 4. Effects of vial inversion on JJAV analysed using SDS-PAGE with silver stain (A), SDS-PAGE Immunoblot (B), AU-PAGE with silver stain (C), and AU-PAGE Immunoblot (D) methodologies. JJAV APIs (1 mg/mL), protected from light and stored refrigerated at 4°C, were kept in inverted position to ensure full exposure to the inner surface of grey butyl rubber stoppers for various period of time. For figures (A) and (B), lanes: 1. MW standards, 2. Baseline sample, 3. Treatment sample incubated for 6 hours, 4. Treatment sample incubated for 24 hours, 5. Treatment sample incubated for 48 hours, 6. JJAV IHR. For figures (C) and (D), lanes: 1. JJAV IHR, 2. Baseline sample, 3. Treatment sample incubated for 6 hours, 4. Treatment sample incubated for 24 hours, and 5. Treatment sample incubated for 48 hours.



Supplemental Figure 5. Effects of artificial light and storage temperatures on JJAV (1 mg/mL) as analysed by SDS-PAGE with silver stain (A), SDS-PAGE Immunoblot (B), AU-PAGE with silver stain (C), and AU-PAGE Immunoblot methodologies. For figures (A) and (B), lanes: 1. MW standards, 2. Baseline sample, 3. Treatment sample incubated at 4°C with no light for 24 hours, 4. Treatment sample incubated at 4°C with artificial light for 24 hours, 5. Treatment sample incubated at 4°C with no light for 48 hours, 6. Treatment sample incubated at 4°C with artificial light for 48 hours, 7. Baseline sample, 8. Treatment sample incubated at 25°C with no light for 24 hours, 9. Treatment sample incubated at 25°C with artificial light for 24 hours, 10. Treatment sample incubated at 25°C with no light for 48 hours, 11. Treatment sample incubated at 25°C with artificial light for 48 hours, and 12. JJAV IHR. For figures (C) and (D), lanes: 1. JJAV IHR, 2. Baseline sample, 3. Treatment sample incubated at 4°C with no light for 24 hours, 4. Treatment sample incubated at 4°C with artificial light for 24 hours, 5. Treatment sample incubated at 4°C with no light for 48 hours, 6. Treatment sample incubated at 4°C with artificial light for 48 hours, 7. Baseline sample, 8. Treatment sample incubated at 25°C with no light for 24 hours, 9. Treatment sample incubated at 25°C with artificial light for 24 hours, 10. Treatment sample incubated at

58 25°C with no light for 48 hours, and 11. Treatment sample incubated at 25°C with
59 artificial light for 48 hours.
60