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Stability of *Myrmecia pilosula* (Jack Jumper) Ant venom for use in immunotherapy

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ABSTRACT

Allergy to *Myrmecia pilosula* (Jack Jumper Ant) venom is common in Australia, affecting ~2.7% of some communities. Venom immunotherapy is a highly effective treatment, but for the venom to be widely distributed for clinical use, the stability and shelf-life of formulated Jack Jumper Ant venom must be demonstrated. HPLC–UV, ELISA Inhibition, SDS-PAGE and SDS-PAGE Immunoblot were used to assess venom stability under conditions of varying temperature, pH and in the presence of various stabilising agents. Optimal stability occurred between pH 8 and 10, however the presence of benzyl alcohol within this pH range resulted in a cloudy appearance within 3 days, so a pH of 6 was used. Increasing polysorbate 80 concentrations accelerated the degradation of allergenic peptides in 100 μg/mL venom, but improved stability at concentrations of 1 μg/mL or less. Sucrose reduced degradation of allergens Myr p 1 and Myr p 3, whilst glycerol was destabilising. In the presence of 22% sucrose, 1.1 mg/mL Jack Jumper Ant venom was stable at −18 °C and 4 °C for 12 months; following dilution to 100 μg/mL with 0.9% sodium chloride, 10 mM phosphate (pH 6), 0.05% polysorbate 80 and 0.9% benzyl alcohol (giving 2% sucrose), venom was stable for 7 days when stored at 4 °C. Concentrated Jack Jumper Ant venom can be stored in 22% sucrose for 12 months, and after dilution to 100 μg/mL for clinical use, it should be discarded after 7 days.

1. Introduction

Jack Jumper ants (*Myrmecia pilosula*, JJA) are a major cause of anaphylaxis in outer urban and rural areas of South-Eastern Australia, including Tasmania, Victoria, southern New South Wales and the cooler areas of South Australia [1]. The incidence of JJA sting allergy is 2.4–2.7% of the adult population in susceptible areas [2,3], with 1.0% of the population having experienced potentially life-threatening symptoms following a sting [3]. Our group has demonstrated the efficacy of Venom Immunotherapy (VIT) in preventing systemic hypersensitivity reactions to JJA stings, reducing the rate of reactions from 72 to 3% ($p < 0.0001$) [4]. Diagnostic skin testing and the initial stages of JJA VIT require very low doses of allergen (0.00001 μg of total protein in some cases), increasing to a usual maintenance dose of 50–100 μg.

JJA venom (JJAV) and its allergenic components have been well characterised [5–7]. The only major allergen is Myr p 2, a 5608 Da heterodimer which has also been called pilosulin 3. Seven minor allergens have been identified, including Myr p 1 and Myr p 3. Myr p 1 occurs as pilosulin 1 (Myr p 1.0101, 6052 Da) and the more predominant [Ile^5]pilosulin 1 (Myr p 1.0102, 6067 Da), whilst other sub-sequences of Myr p 1 have been identified [8]. Myr p 3 is expressed as the homodimer pilosulin 4.1, which has a molecular mass of 8212 Da. Four "higher molecular weight" allergens ranging in size from 22.6 to 89.8 kDa have been identified, but remain unnamed as the cDNA or amino acid sequence have not been determined [7].

Allergen products undergo extensive standardisation prior to release, but they may contain numerous components that are unstable, and ensuring their ongoing stability is as important as the initial standardisation process. Compounds that have been added in an attempt to improve allergen stability in aqueous solutions include sugars that inhibit local protein unfolding, agents such as human albumin and polysorbate 80 that reduce allergen adsorption and microbiological preservatives [9,10]. The stability of some grass, pollen, yeast, animal, mite and food allergen extracts

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have been described [9–12]; however, there is little published data regarding the stability of any Hymenoptera venom preparations other than in product information sheets.

The stability of allergen products can assessed by RAST or ELISA inhibition which determines total allergenic potency but provides no information on individual allergen content, whereas two-site ELISA assays can quantify individual allergen content, but not allergenic potency. SDS-PAGE, isoelectric focussing and crossed-immunoelectrophoresis allow for qualitative or semi-quantitative analysis of individual proteins, and immunoblotting allows observation of IgE-binding components.

Formulation of products for use in diagnosis and treatment of allergic diseases requires a number of considerations that are relatively unique amongst pharmaceutical preparations:

- Allergen preparations contain a number of peptides and proteins which must be present in sufficient quantities for the product to be deemed stable and which may require different conditions for optimal stability.
- Serial dilutions are required during diagnosis and treatment and a simple method of presenting and diluting the product is required to minimise the risk of dosing errors that may lead to serious adverse events for patients [13,14]. Stability must be confirmed at each of the concentrations used clinically.
- Various storage conditions are required for different dilutions of an allergen product—long term storage at −18 °C or 4 °C is possible for concentrated solutions or freeze dried products, but more dilute solutions are prepared at the bedside where refrigeration is not always practicable.
- Freeze drying is used to stabilise a number of peptide and protein pharmaceuticals, but it is not economically viable for some allergen products with a relatively small market.

Furthermore, the tests used to standardise and determine the stability of allergenic products are less precise than the tests used with small molecule pharmaceuticals, and the complex nature of allergenic products means that the usual criteria for stability indicating assay methods are not appropriate. What is important is that total allergenic potency and the quantity of the most important allergens are maintained for the duration of the shelf-life of a product.

The protein and allergen composition and methods to standardise JJAV have been well defined [5–7,15]. Using these HPLC–UV, ELISA Inhibition and SDS-PAGE/SDS-PAGE Immunoblot methods, our aims were to define the optimal conditions for ensuring the stability of JJAV during distribution and clinical use, and to determine the shelf-life of venom under typical conditions of use.

2. Materials and methods

2.1. Materials

All materials used for JJAV analysis have been described previously [15]. Chemicals used for venom formulation were of United States, British or European Pharmacopoeia standard. Benzyl alcohol and sucrose were obtained from MP Biomedicals (Solon, Ohio), sodium chloride and water from Astra Zeneca (Perth, Australia), polysorbate 80 from Merck (Darmstadt, Germany), phenol and glycerol from David Craig (Melbourne, Australia) and sodium phosphate dibasic dihydrate, sodium phosphate monobasic dihydrate and ε-aminocaproic acid from Sigma–Aldrich (St Louis, Missouri). Venom and diluent solutions were stored in United States Pharmacopoeia Type I glass vials and sealed with grey chlorobutyl rubber stoppers (Wheaton Science Products, Millville, New Jersey).

2.2. Jack Jumper Ant venom

JJAV was obtained from ants collected from a variety of locations around Tasmania by venom sac dissection as described previously [5]. The protein content was determined by comparison with In-House Reference venom of known concentration. It was subsequently analysed by ELISA Inhibition to determine allergenic potency, and individual allergen content was determined by HPLC–UV, SDS-PAGE and SDS-PAGE Immunoblot [15].

2.3. Analysis of stability test samples

The minimum concentration of venom that allowed accurate analysis of JJAV by HPLC–UV, ELISA Inhibition, SDS-PAGE (and SDS-PAGE Immunoblot) was 100 μg/mL, 0.1 μg/mL and 1 mg/mL respectively. Analyses were performed as described previously [15]. For HPLC–UV analysis of 1 mg/mL samples, 7.5 μg of venom protein was injected onto the HPLC column, and for 100 μg/mL solutions, 50 μL (containing 5 μg venom protein) was used. All ELISA Inhibition and SDS-PAGE Immunoblot experiments used the same batch of pooled positive serum, and for SDS-PAGE and SDS-PAGE Immunoblot analysis, 10 μg and 4 μg of venom protein respectively were loaded per lane; samples containing 100 μg/mL venom were concentrated to 1 mg/mL using a centrifugal filtration device (Microcon® 3 kDa nominal molecular weight cut-off, Millipore, Billerica, Maryland, USA) prior to analysis [16]. Venom was not concentrated prior to HPLC–UV or ELISA Inhibition, as this process resulted in at least 25% protein loss (data not shown). As such, ELISA Inhibition was the only analysis performed on samples with venom concentration between 10 μg/mL and 0.1 μg/mL.

2.4. Control samples and data analysis

Baseline and/or concurrent control samples were included in all stability trials and stored at −80 °C until analysis. When analysing the HPLC–UV and ELISA Inhibition data, values from a stability trial sample were compared to the mean value obtained from the baseline/control sample(s). Results for HPLC–UV analysis are expressed as a percentage of the baseline/control value, and ELISA Inhibition as the relative allergenic potency, expressed as a percentage of the potency of the baseline/control value. SDS-PAGE and SDS-PAGE Immunoblot experiments were analysed qualitatively and semi-quantitatively, and baseline/control samples were included in all experiments.

2.5. Stability trial conditions

Venom in the various stability studies described below was stored within three temperature ranges; room temperature (20–25 °C), refrigerator temperature (4–8 °C) and freezer temperature (−20 to −18 °C).

2.5.1. General approach

The storage conditions used for the stability studies were largely chosen in an attempt to reflect use of the products in clinical practice. The ionic strength of solutions intended for administration (i.e. 100 μg/mL and below) was approximately isotonic, but slight variations were tolerated if this facilitated simplicity of presentation and/or preparation for clinical use. Concentrated solutions must be diluted prior to use and were stored in frozen or refrigerated conditions. Once diluted for clinical use, solutions that were stable for at least 24 h may have been transferred to the refrigerator following administration so that the remaining portion could be used at a subsequent visit, whereas other dilutions were less stable (i.e. degraded within 24 h), so refrigeration was impracticable and/or unreliable in the clinic. As such, the stability of different

dilutions was investigated at one or both of room or refrigerated temperatures.

The effect of stabilising excipients on the stability of concentrated (1 mg/mL) JJAV was initially defined and, subsequently, the effect of pH on stability of 100 μg/mL venom investigated. The effect and optimal concentration of polysorbate 80 (in more dilute venom concentrations where its effect at reducing surface adsorption was likely to be most pronounced) and addition of benzyl alcohol was then examined. Finally, the stability of the chosen formulations was determined and shelf-lives allocated.

2.6. Individual stability studies

2.6.1. The effect of freeze–thaw cycles

Aliquots of 1 mg/mL JJAV in water were put through a cycle of being snap frozen at −80 °C and then thawed for a total of 5, 10 and 20 cycles. A control sample was frozen once then thawed prior to analysis. Samples were analysed by HPLC–UV, ELISA Inhibition, SDS-PAGE and SDS-PAGE Immunoblot.

2.6.2. The effect of glycerol, sucrose and ε-aminocaproic acid on venom stability

JJAV (1 mg/mL) was stored at −18 °C and 4 °C for 12 months in either 0.9% sodium chloride, 6.6 M (50%, v/v) glycerol, 1 M (34.2%, w/v) sucrose or 1 M (13.2%, w/v) ε-aminocaproic acid. Samples were taken after 0, 3, 6 and 12 months for analysis by HPLC–UV, SDS-PAGE and SDS-PAGE Immunoblot.

2.6.3. The effect of pH on venom stability

Solutions of 0.9% sodium chloride were adjusted to pH of 4, 5, 6, 7, 8, 9 and 10 with hydrochloric acid and sodium hydroxide (trace amounts of phosphate or carbonate were included as buffers to allow precise pH manipulation) and used to dilute JJAV to 100 μg/mL. Venom was stored at 4 °C and samples were taken after 0, 1, 2, 4 and 12 weeks for HPLC–UV analysis.

2.6.4. The effect of polysorbate 80 concentration on venom stability

2.6.4.1. JJAV (100 μg/mL) at room temperature. Solutions containing 0.9% sodium chloride, 10 mM phosphate buffer (pH 6) and polysorbate 80 spanning a range of concentrations (0, 0.0001, 0.0005, 0.001, 0.005, 0.01 and 0.05%, v/v) were used to dilute venom to 100 μg/mL prior to storage. Venom was protected from light and stored at room temperature to accelerate any degradation of venom peptides, and samples were taken after 0, 4 and 10 days for HPLC–UV analysis.

2.6.4.2. Immediate stability of 0.1 μg/mL, 1 μg/mL and 10 μg/mL JJAV. Various amounts of polysorbate 80 (to produce concentrations of 0, 0.0005, 0.005 and 0.05%, v/v) were included with diluent solution containing 0.9% sodium chloride and 10 mM phosphate buffer (pH 6). A solution containing 100 μg/mL venom was made in each diluent solution, then successive 10-fold dilutions prepared by adding 0.2 mL aliquots to 1.8 mL vials of diluent solution to prepare samples containing 10 μg/mL, 1 μg/mL and 0.1 μg/mL. Samples were analysed via ELISA Inhibition and the allergenic potency of the diluted samples was compared to the mean 50% Inhibition value obtained from the 100 μg/mL samples.

2.6.5. Addition of benzyl alcohol as a microbiological preservative

JJAV was adjusted to 100 μg/mL in solutions that contained 10 mM phosphate buffer (pH 6, 7 and 8), 0.05% polysorbate 80, 0.9% (v/v) benzyl alcohol and either 0.9% sodium chloride, 245 mM (isotonic) sucrose or 0.9% sodium chloride with 2% sucrose. Solutions were stored at room temperature, protected from light, and samples were taken for HPLC–UV analysis after 0, 1, 3 and 7 days.

2.6.6. Stability of JJAV 1.1 mg/mL in 22% sucrose

JJAV was formulated in a solution that contained 1.1 mg/mL venom in 22% sucrose (0.643 M) and 110 μg (100 μL) was transferred to single use glass vials. Aliquots were stored at −18 °C and 4 °C and a vial removed after 1, 3, 6 and 12 months for analysis via HPLC–UV, ELISA Inhibition, SDS-PAGE and SDS-PAGE Immunoblot.

2.6.7. Stability of JJAV 100 μg/mL

JJAV (110 μg in 100 μL of 22% sucrose) was diluted to 100 μg/mL via the addition of 1 mL diluent solution (containing 0.9% sodium chloride, 10 mM phosphate buffer (pH 6), 0.05% polysorbate 80 and 0.9% benzyl alcohol) and stored, protected from light, at either 4 °C or room temperature. Samples were taken after 0, 1, 3, 7 and 14, 28 and 42 days (28 and 42 days samples were not taken for the sample stored at room temperature). Analysis was via HPLC–UV, ELISA Inhibition, SDS-PAGE and SDS-PAGE Immunoblot.

2.6.8. Stability of 10 μg/mL, 1 μg/mL and 0.1 μg/mL JJAV

100 μg/mL JJAV was prepared from JJAV solution (1.1 mg/mL in 22% sucrose) by adding diluent solution (containing 0.9% sodium chloride, 10 mM phosphate buffer (pH 6), 0.05% polysorbate 80 and 0.9% benzyl alcohol) and then diluting 10-fold successively by adding 0.2 mL aliquots to 1.8 mL vials of diluent solution to prepare samples containing 10 μg/mL, 1 μg/mL and 0.1 μg/mL. Solutions were stored at room temperature and samples were taken after 0, 2, 6 and 24 h for analysis via ELISA Inhibition. The 50% Inhibition value of the stored sample was compared to the mean 50% Inhibition value for the baseline sample containing 100 μg/mL.

3. Results

3.1. The effect of freeze–thaw cycles

No effect of up to 20 freeze–thaw cycles was apparent using any of the analytical methods.

3.2. The effect of glycerol, sucrose and ε-aminocaproic acid on venom stability

At −18 °C, HPLC–UV data indicated that under all of the conditions tested, the amount of Myr p 1, Myr p 2 and Myr p 3 was equivalent and SDS-PAGE and corresponding Immunoblots did not show any noticeable differences in total protein profile or IgE-binding bands. In contrast, HPLC–UV analysis of JJAV stored in 50% glycerol at 4 °C revealed marked destabilisation of all allergenic peptides, which was not accompanied by new peak development. SDS-PAGE and SDS-PAGE Immunoblot analysis supported this, the results suggesting that peptide aggregation had occurred (data not shown). The stability of Myr p 2 was equivalent in 0.9% sodium chloride and 1 M sucrose, but sucrose delayed the degradation of Myr p 3 and Myr p 1 (data not shown). When ε-aminocaproic acid was used as a stabilising agent, HPLC–UV analysis indicated that the levels of Myr p 2 and Myr p 1 increased during storage, whilst Myr p 3 disappeared within 6 months. SDS-PAGE and SDS-PAGE Immunoblot did not reveal any significant differences in venom stability when sodium chloride, sucrose or ε-aminocaproic acid were included in the formulation. Given these results, sucrose was chosen as a stabilising agent for subsequent formulations containing high concentrations of venom (i.e. >1 mg/mL).

3.3. The effect of pH on venom stability

Myr p 1.0102 was the least stable of the allergenic peptides; after 4 weeks of storage, it was completely absent at pH ≤7. Its degradation was accompanied by an increase in the peak with a retention

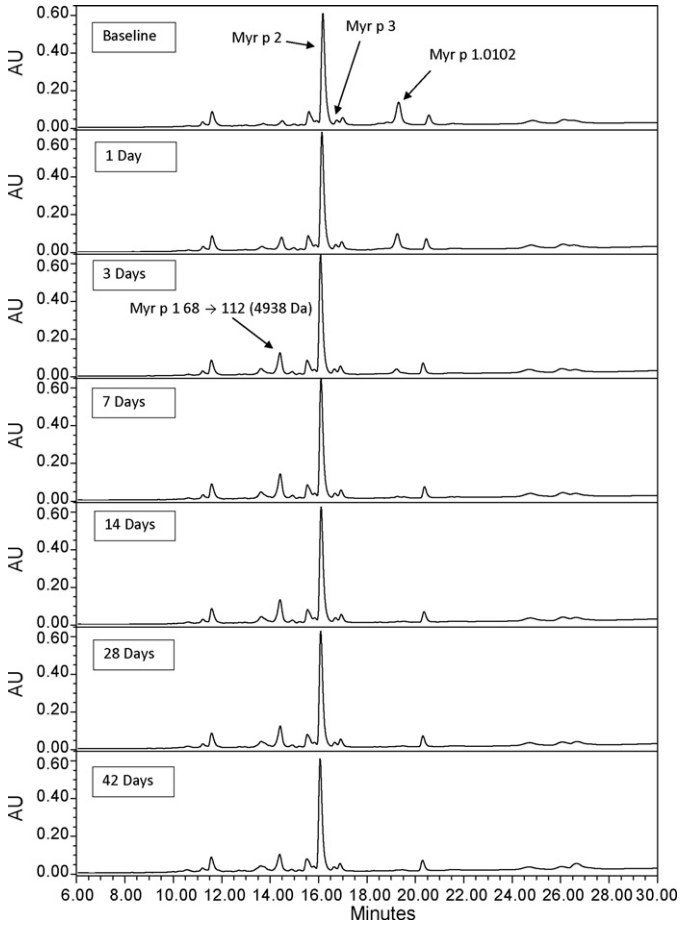

Fig. 1. HPLC–UV profile of 100 μg/mL JJAV stored at 4 °C for 42 days (Section 2.6.7). Samples were analysed by HPLC–UV at baseline and after 1, 3, 7, 14, 28 and 42 days as annotated on the chromatograms. Myr p 1.0102 (elution time 19.4 min and annotated in baseline sample) degrades during the first 7 days of storage and there is a corresponding increase in a peak with elution time of 14.5 min (Myr p 1 68 → 112, annotated in 3-day sample). Myr p 2 and Myr p 3 are annotated in the baseline sample, and other peaks are peptides that have not been identified as JJAV allergens.

time of approximately 14.5 min—previously identified as a breakdown product of Myr p 1.0102 with molecular mass 4938 Da (Myr p 1 68 → 112) [5]. Retrospective analysis of assay validation data showed that the ratio of this HPLC–UV peak area to total amount of venom analysed was linear (unpublished). A series of HPLC–UV chromatograms displaying this change is shown in Fig. 1. Donovan et al. [17] has shown that the IgE-binding domain of Myr p 1 occurs between residues 93 → 106; given that both peptides contain this sub-sequence, it is assumed they have equivalent IgE-binding activity. The total amount of Myr p 1 was therefore calculated by adding the areas of Myr p 1.0102 and Myr p 1 68 → 112 and compared to the total amount of Myr p 1.0102 and Myr p 1 68 → 112 in the baseline/control sample. From hereon, the term Myr p 1 will be used to describe the sum of the amount of Myr p 1.0102 and its 4938 Da (Myr p 1 68 → 112) degradation product. The stability of the three allergens quantifiable by HPLC–UV at each pH is shown in Table 1.

3.4. The effect of polysorbate 80 concentration on venom stability

HPLC–UV analysis indicated that increased concentrations of polysorbate 80 lead to more rapid degradation of Myr p 1, Myr p 2 and Myr p 3 in 100 μg/mL JJAV (Table 2). Conversely, at concentrations of 0.1–10 μg/mL, 0.05% polysorbate 80 was required to maintain the allergic potency of venom following mixing in glass vials (Table 3), so this concentration of polysorbate 80 was used for future studies.

Table 1
Stability of JJAV allergens at various pH after storage at 4 °C for 12 weeks (Section 2.6.3).

pH	Percentage of Myr p 1 present compared to baseline			
	Storage time (weeks)			
	1	2	4	12
4	63.0%	68.9%	57.4%	22.9%
5	53.0%	46.1%	10.9%	0.0%
6	30.5%	0.0%	0.0%	0.0%
7	61.7%	15.2%	0.0%	0.0%
8	62.4%	54.9%	49.2%	27.9%
9	62.3%	57.5%	49.7%	30.9%
10	52.4%	50.8%	42.4%	47.8%
pH	Percentage of Myr p 2 present compared to baseline			
	Storage time (weeks)			
	1	2	4	12
4	82.7%	57.1%	26.2%	1.0%
5	77.6%	53.4%	6.9%	0.9%
6	71.2%	41.7%	10.1%	0.0%
7	65.9%	51.2%	31.8%	0.9%
8	73.5%	71.3%	68.4%	64.1%
9	73.4%	72.2%	66.4%	63.1%
10	65.4%	68.9%	70.5%	60.1%
pH	Percentage of Myr p 3 present compared to baseline			
	Storage time (weeks)			
	1	2	4	12
4	60.9%	56.6%	58.3%	0.0%
5	56.6%	59.5%	57.2%	0.0%
6	56.8%	56.7%	38.6%	0.0%
7	62.7%	61.0%	55.6%	20.4%
8	69.6%	66.6%	65.5%	57.1%
9	71.9%	77.8%	63.7%	53.9%
10	62.7%	75.7%	73.1%	47.1%

JJAV was stored between pH 4 and 10, and samples were taken at baseline, and after 1, 2, 4 and 12 weeks and analysed by HPLC–UV.

3.5. Addition of benzyl alcohol as a microbiological preservative

After 3 days of storage, all solutions stored at pH 7 and 8 developed cloudiness with the exception of the 245 mM sucrose solution at pH 8. The 245 mM sucrose at pH 6 and 8 subsequently appeared cloudy after 7 days. Phenol was not considered for use as a microbiological preservative, because a preliminary experiment showed that its inclusion in solutions containing 0.9% sodium chloride and

Table 2
Effect of polysorbate 80 concentration on stability of allergenic peptides in 100 μg/mL JJAV (Section 2.6.4.1).

Polysorbate 80 concentration (%, v/v)	Percentage of allergen present compared to baseline					
	Myr p 2		Myr p 3		Myr p 1	
	4 Days	10 Days	4 Days	10 Days	4 Days	10 Days
0	103.5%	74.4%	88.0%	52.7%	92.3%	63.1%
0.0001	93.7%	74.4%	70.7%	53.5%	90.8%	63.8%
0.0005	87.9%	82.0%	69.6%	58.6%	85.7%	74.7%
0.001	82.6%	70.4%	63.4%	51.2%	84.8%	69.5%
0.005	63.5%	52.2%	18.4%	0.0%	79.4%	67.6%
0.01	66.1%	24.6%	0.0%	0.0%	85.2%	59.9%
0.05	58.1%	7.0%	0.0%	0.0%	82.4%	26.9%

Venom was stored in 0.9% sodium chloride and 10 mM phosphate (pH 6) at room temperature in various concentrations of polysorbate 80 (0–0.05%). Samples were taken for analysis by HPLC–UV at baseline, and after 4 and 10 days.

Table 3
Relative allergenic potency of JJAV after dilution to 10 μg/mL, 1 μg/mL and 0.1 μg/mL in the presence of 0–0.05% polysorbate 80 (Section 2.6.4.2).

Venom concentration (μg/mL)	Allergenic potency compared to baseline			
	Polysorbate 80 concentration (%, v/v)			
	0	0.0005	0.005	0.05
100	70.7%	143.1%	83.7%	144.3%
10	30.9%	59.9%	62.3%	154.8%
1	<20%	<20%	44.3%	117.5%
0.1	<20%	<20%	29.7%	89.5%

JJAV was diluted to 100 μg/mL with diluent solution containing 0.9% sodium chloride, 10 mM phosphate buffer (pH 6) and 0–0.05% polysorbate 80, and serial dilutions to 10 μg/mL, 1 μg/mL and 0.1 μg/mL were prepared. Samples were taken immediately for analysis of allergenic potency by ELISA Inhibition.

0.05% polysorbate 80 (at pH 6, 7, 8 and 9) caused cloudiness to appear immediately. Benzyl alcohol and phosphate buffer (pH 6) was therefore used in subsequent stability trials.

3.6. Stability of JJAV 1.1 mg/mL in 22% sucrose

HPLC–UV and ELISA Inhibition analyses of the samples stored at −18 °C and 4 °C are shown in Table 4. The relative amount of Myr p 3 increased to 193.7% when stored at 4 °C after 12 months, whereas all other peptides were within 82.3–136% of baseline throughout the 12-month period during storage at both −18 °C and 4 °C. SDS-PAGE and SDS-PAGE Immunoblot were identical for all samples (data not shown).

3.7. Stability of JJAV 100 μg/mL

At room temperature, HPLC–UV analysis showed that the amount of Myr p 1, Myr p 2 and Myr p 3 declined over the 14-day study period to 42.1, 77.1 and 59.8% of the baseline value respectively, whilst at 4 °C, the amount of Myr p 1, Myr p 2 and Myr p 3 declined over the 42-day study period to 52.6, 92.5 and 74.6% respectively (Fig. 1 and Table 5). ELISA Inhibition indicated that the relative allergenic potency was slightly increased for all stability test samples, and remained between 98.8 and 164.2% of the baseline value (Table 5). SDS-PAGE analysis of the samples stored at 4 °C and room temperature were similar, but the immunoblots showed that the 25.6 kDa allergen appeared to progressively decrease in molecular weight throughout the study period in both the 4 °C and room temperature samples (the 4 °C sample is shown in Fig. 2). This happened immediately (i.e. in the baseline sample following dilution with polysorbate 80), and after 7 days, the band had completely disappeared, but was accompanied by the formation of IgE-binding bands with molecular weight of approximately 24, 22 and 20 kDa. These bands appeared to quantitatively bind IgE at a similar level to the native 25.6 kDa band.

3.8. Stability of 10 μg/mL, 1 μg/mL and 0.1 μg/mL JJAV

The allergenic potency of JJAV stored at 10 μg/mL increased to 156.3% of the baseline value over the 24-h study period, whereas the 1 μg/mL decreased to 77.5% potency. The 0.1 μg/mL sample rapidly lost allergenic potency during the study period, and its allergenic potency was 21.5% of the baseline value after 4 h of storage (Table 6).

4. Discussion

By manipulating the storage conditions, 1.1 mg/mL JJAV formulated with 22% sucrose and stored at −18 °C or 4 °C is stable for 12 months, and once diluted to 100 μg/mL with a solution contain-

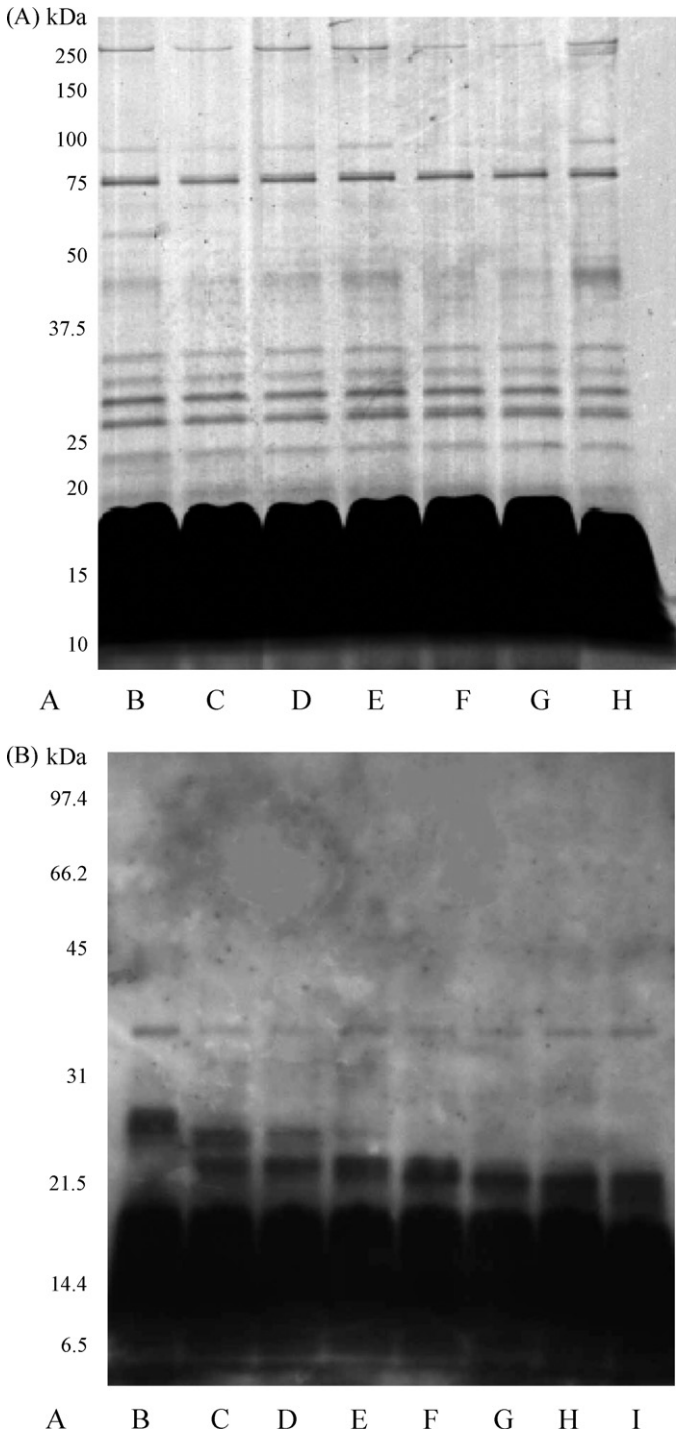

Fig. 2. SDS-PAGE (A) and SDS-PAGE Immunoblot (B) of JJAV (100 μg/mL) stored at 4 °C for 42 days (Section 2.6.7). JJAV was diluted to 100 μg/mL in a solution that also contained 2% sucrose, 0.9% sodium chloride, 10 mM phosphate buffer (pH 6), 0.05% polysorbate 80 and 0.9% benzyl alcohol. Lane markers in SDS-PAGE image (image A) represent samples that were taken at baseline (lane B) and after 1 (lane C), 3 (lane D), 7 (lane E), 14 (lane F), 28 (lane G) and 42 (lane H) days, concentrated to 1 mg/mL and analysed by SDS-PAGE. Lane A represents molecular weight markers. In the SDS-PAGE Immunoblot image (image B), lane markers represent samples that were taken pre-dilution (lane B), at baseline (lane C) and after 1 (lane D), 3 (lane E), 7 (lane F), 14 (lane G), 28 (lane H) and 42 (lane I) days, concentrated to 1 mg/mL, and analysed by SDS-PAGE Immunoblot. Lane A represents molecular weight markers.

Table 4
Stability of JJAV (110 μg/0.1 mL) after storage at −18 °C and 4 °C in 22% sucrose for 12 months (Section 2.6.6).

Duration of storage (months)	Percentage compared to baseline							
	Myr p 2		Myr p 3		Myr p 1		Allergenic potency	
	−18 °C	4 °C	−18 °C	4 °C	−18 °C	4 °C	−18 °C	4 °C
1	104.5%	102.9%	111.6%	85.8%	89.9%	91.3%	118.2%	148.4%
3	82.3%	103.8%	136.0%	183.2%	88.9%	92.3%	128.9%	105.3%
6	99.5%	94.0%	91.9%	135.8%	101.7%	93.5%	102.7%	104.4%
12	99.0%	98.6%	98.6%	193.7%	102.1%	94.3%	105.5%	119.0%

JJAV was formulated in aliquots of 110 μg/0.1 mL in 22% sucrose and stored at −18 °C and 4 °C. Samples were taken for analysis after 1, 3, 6 and 12 months and the relative content of Myr p 1, Myr p 2 and Myr p 3 determined by HPLC–UV and allergenic potency by ELISA Inhibition.

Table 5
Stability of JJAV (100 μg/mL) stored at 4 °C or room temperature for 42 days (Section 2.6.7).

Time (days)	Percentage compared to baseline							
	Myr p 2		Myr p 3		Myr p 1		Allergenic potency	
	4 °C	Room temperature	4 °C	Room temperature	4 °C	Room temperature	4 °C	Room temperature
0	97.9%	102.1%	97.7%	102.3%	98.7%	101.3%	98.8%	101.2%
1	100.2%	98.9%	107.4%	96.1%	104.0%	84.4%	110.5%	164.2%
3	99.3%	96.2%	100.3%	93.0%	85.4%	73.0%	134.4%	146.6%
7	98.7%	92.0%	90.5%	91.1%	76.2%	59.4%	135.9%	121.8%
14	97.2%	77.1%	90.5%	59.8%	70.9%	42.1%	146.6%	125.0%
28	96.0%	Not assessed	84.7%	Not assessed	64.5%	Not assessed	153.4%	Not assessed
42	92.5%	Not assessed	74.6%	Not assessed	52.6%	Not assessed	141.8%	Not assessed

JJAV was diluted to 100 μg/mL in a solution that also contained 2% sucrose, 0.9% sodium chloride, 10 mM phosphate buffer (pH 6), 0.05% polysorbate 80 and 0.9% benzyl alcohol and samples were taken at baseline and after 1, 3, 7, 14, 28 and 42 days and the relative content of Myr p 1, Myr p 2 and Myr p 3 determined by HPLC–UV and allergenic potency by ELISA Inhibition.

ing 0.9% sodium chloride, 10 mM phosphate buffer (pH 6), 0.05% polysorbate 80 and 0.9% benzyl alcohol, is stable for a further 7 days. Solutions containing 10 μg/mL JJAV are stable for 24 h at room temperature and those diluted to 0.1 μg/mL and 1 μg/mL should be used within 2 h of preparation. No stability data is available for more dilute solutions, so these must be used immediately and then discarded.

During storage of 100 μg/mL JJAV, a presently uncharacterised 25.6 kDa minor allergen disappeared rapidly and appeared to be replaced with new IgE-binding bands with lower molecular weight. These bands (molecular weight 20, 22 and 24 kDa) semi-quantitatively recognise a similar amount of IgE as the 25.6 kDa band (Fig. 2, image B), and it appears that this represents a similar situation to the degradation of Myr p 1.0102, where allergenic activity is maintained despite protein cleavage. There may also be concurrent aggregation of lower molecular weight allergens that also bind IgE and are responsible for the 20 kDa band. To be cautious, we have deemed the 100 μg/mL JJAV formulation as stable for as long as the 22 kDa IgE-binding band was apparent (i.e. 7 days), although the significant epitopes derived from the 25.6 kDa allergen may persist in sufficient quantities for longer.

Table 6
Short-term stability of 10 μg/mL, 1 μg/mL and 0.1 μg/mL JJAV solutions (Section 2.6.8).

Duration of storage (h)	Allergenic potency compared to baseline		
	JJAV concentration (μg/ml)		
	10	1	0.1
0	105.3%	103.1%	92.6%
2	116.3%	78.1%	63.7%
6	149.3%	84.7%	21.5%
24	156.3%	77.5%	<20%

JJAV (110 μg/0.1 mL of 22% sucrose) was diluted to a venom concentration of 100 μg/mL with diluent solution containing 0.9% sodium chloride, 10 mM phosphate buffer (pH 6), 0.05% polysorbate 80 and 0.9% benzyl alcohol, then serially diluted to 10 μg/mL, 1 μg/mL and 0.1 μg/mL with the same diluent solution. Samples were taken immediately, and after 2, 6 and 24 h for analysis by ELISA Inhibition.

In comparison to JJAV, venoms from honey bee, European and paper wasp that are commercially available in Australia appear to have longer shelf-lives. These formulations are freeze dried with mannitol, and following reconstitution with a solution of 0.03% human albumin, 0.9% sodium chloride and 0.4% phenol, the 100 μg/mL solution has an expiry of 6 months, the 10 μg/mL and 1 μg/mL solutions 1 month, the 0.1 μg/mL solution 14 days, whilst more dilute solutions should be made fresh and discarded immediately. The methods used to allocate these shelf-lives have not been published and shelf-lives have not been modified since the identification of novel allergens in honeybee and paper wasp venoms [18,19]. All of the important allergens may therefore not be present at appropriate concentrations during these prolonged storage periods.

Guidelines for allergen stability differ between the US and European Regulatory Agencies. In the US, guidelines for the stability testing of grass pollen extracts published by the Centre for Biologics Evaluation and Research (CBER) state that, at each time point of the stability test, the allergenic potency of the extract must be identical to the reference extract. In contrast, European guidelines state that no less than 30% of the stated allergenic activity should be maintained at the end of shelf-life [20]. In standardised allergen preparations, the European Pharmacopoeia specifies that the allergenic potency and absolute amount of all major and some minor allergens is between 50 and 200% of a reference preparation, whereas in the US, in house testing must conform to tighter limits prior to testing at CBER, where release limits are 50 and 200% of their internal standard. Given these two related guidelines, there is the potential for large differences in specific allergen levels and relative allergenic potency in products stored for the duration of the shelf-life. We have applied a hybrid of the US and European definitions of stability, where JJAV must have allergenic potency and quantity of Myr p 1, Myr p 2 and Myr p 3 within 50 and 200% of the baseline preparation and SDS-PAGE and SDS-PAGE Immunoblots appear identical to baseline samples.

The European guidelines do not consider the potential for allergenic potency to increase during storage, as was the case with 100 μg/mL JJAV; this increase in potency was noted immediately after addition of diluent solution. JJAV peptide allergens are amphiphilic [21], and other amphiphilic peptides such as melittin have the potential to form aggregates, particularly at high ionic strength (i.e. 0.9% sodium chloride) [22]. This may in effect, 'hide' allergenic sub-sequences so that they are unable to bind allergic IgE. The polysorbate 80 within diluent solution reduces surface tension, which may promote dissociation of these aggregates and explain why allergenic potency increased after its addition. Furthermore, peptides may be partially protected from degradation whilst in these aggregates, explaining why increasing polysorbate 80 concentration within diluent solution was associated with worsening stability of 100 μg/mL JJAV. At lower venom concentrations however, surface adsorption appeared to be the primary mode of instability, necessitating a relatively high concentration of polysorbate 80 in diluent solution.

The main strength of this study is the large number of complementary analytical methods used in an attempt to overcome the weaknesses inherent to each individual method. SDS-PAGE and SDS-PAGE Immunoblot were used to qualitatively observe the higher molecular weight allergens, but is of limited value in assessing the lower molecular weight peptide allergens; HPLC–UV was able to determine the stability of these lower molecular weight allergens. The use of HPLC–UV, correlated to previous HPLC–mass spectrometry analysis [5] allowed the relative quantity and exact identity of each peak to be determined in a single assay. If the IgE-binding domain(s) of an allergen are known, HPLC–UV has the potential to quantify the contribution of degradation products that also contain the IgE-binding domain to total allergen quantity, as is the case with Myr p 1 in this study.

Given the significant protein loss during centrifugal filtration, the only quantitative assay that could be performed for venom at a concentration between 0.1 μg/mL and 10 μg/mL was ELISA Inhibition, and no testing was possible at venom concentrations below 0.1 μg/mL. Furthermore, when using pooled allergic sera for ELISA Inhibition, changes in specific allergen potency could not be determined. Whilst HPLC–UV accurately quantitates some individual allergens, it is unable to detect minor components within the entire mixture such as the 25.6 kDa allergen. Furthermore, some degradation products may have similar retention times to native allergens, leading to inaccurate results. For example, the apparent increase in Myr p 3 concentration during storage in 22% sucrose at 4 °C may be due to the formation of a minor degradation product with similar retention time.

JJAV is not a registered pharmaceutical agent, and is unlikely to ever be commercially available; it is only used by a small number of allergists who work in areas where JJA is endemic. As such, the consistency or robustness of the stability data has not been assessed to the level that would be required if the product was intended for commercial use. For example, multiple replicates of the final stability studies and analysis of the effect of inverting the vial (and therefore leaving venom solution in contact with the stopper, which we currently control for by instructing users of the product not to invert the contents of the vial) would need to be performed.

Within this study, JJAV obtained from the island state of Tasmania was used, but ants are endemic in other mainland areas of Australia. Venom was standardised to ensure consistency between batches, but seasonal variability has been noted for allergens in other Hymenoptera venoms [23,24] and there may also be geographical differences in venom composition. Seasonal differences have been accounted for by pooling venom collected at different times, but the significance of variability of JJAV in different areas of Australia is currently the subject of further investigation. These findings will determine if venom from other locations is required to treat allergy in people who do not live in Tasmania.

The 25.6 kDa IgE-binding band was previously shown to be a minor allergen in JJAV [7], so its quantification is not a requirement of the European Pharmacopoeia [25]. However, it is recognised by the sera of 46.3% of allergic individuals (50% of allergic individuals must recognise a protein for it to be a major allergen [26]), so the significance of its degradation in 100 μg/mL JJAV solutions is unclear, and is a limitation of SDS-PAGE Immunoblot using pooled positive sera to determine allergen stability. More informative methods of determining its stability are required, but allergen specific ELISA may not be useful in this situation, as these assays do not reliably detect different isoforms of the same allergen [27,28]. When assessing the stability of dust mite extract, a lack of correlation between the total allergenic potency and the amount of individual allergens determined by specific ELISA was noted [12]. This was due to the inability of the assay to detect all isoforms of the major allergens, which may occur if a similar method is used to assess the 25.6 kDa allergen, where degradation products appear to have equivalent IgE-binding activity.

T-cell epitopes and IgE-binding domains of allergens are not homologous, and the importance of T-cells and T-cell epitopes on the efficacy of VIT is well documented [29]. Whereas T-cell epitopes are important in achieving successful desensitisation, IgE-binding domains can lead to cross-linking and degranulation of basophils and mast cells and toxicity. ELISA Inhibition was used in this study to determine allergenic potency, but this test does not determine the content of T-cell epitopes within an allergen mixture, rather the overall IgE-binding capacity. Whilst we did not attempt to standardise or determine the stability of T-cell epitopes within JJAV, by recognition of specific peptide sequences, HPLC–UV coupled with mass spectrometry has the potential to quantify T-cell epitopes in complex allergen mixtures, and may be a useful tool for allergen standardisation and stability testing in the future. Other techniques that allow determination of T-cell stimulation capacity include T-cell proliferation assays [30] and interleukin-4 ELISPOT [31], both of which are time consuming and require access to T-cells from allergic patients. Identification of the JJAV T-Cell proteome and the development of assays to quantify the number of T-cell epitopes in venom preparations would be a valuable advance. This would facilitate a comparison of HPLC–UV/MS, T-cell stimulation and interleukin-4 ELISPOT assays to quantitate T-cell epitopes in venom preparations.

5. Conclusion

The effect of pH, temperature, type and concentration of various stabilising agents has a dramatic effect on JJAV stability. These are likely to be critical to the stability of other allergens, and for new and/or small market allergen products, this study may provide some guidance for producing a relatively inexpensive and stable formulation.

Conflict of interest

No conflicts of interest are noted.

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