ARQUIVOS DE ASMA, ALERGIA E IMUNOLOGIA

Original Article

Evaluation of the action of precipitating agents to obtain allergenic proteins from crude mite extracts

Avaliação da ação de agentes precipitantes na obtenção de proteínas alergênicas presentes em extratos brutos de ácaros

Francisca das Chagas Sobral Silva[1,2], Daniel Vasconcelos Silva[2], Anderson Bruno Matos[2], Andrea Medeiros Salgado[1], Maria Queiroz da Cruz[2]

ABSTRACT

Introduction: Allergen proteins found in dust mite extracts, such as *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Tyrophagus putrescentiae* (TP), are relevant for scientific studies in the allergy and immunotherapy fields. The precipitation/concentration of protein extracts may favor the aggregation of the allergens in homogenates. **Objective and method:** This paper investigates the precipitation process by submitting crude mite extracts to compounds such as ammonium sulfate $(NH_4)_2SO_4$, trichloroacetic acid (TCA) and acetone. **Results:** The best results were obtained by fractionation with $(NH_4)_2SO_4$ at 80% (w/v) saturation (~0° C), observing the protein markings on the electrophoresis gel. Major allergens were identified by immunoblot at 25 kDa (cysteine protease) for Der f and Der p; and 25 kDa, 30 kDa (tropomyosin) and Try p 3, near 26 kDa. For this percentage the total protein contents were 12.83 mg mL^{-1} for Der f, 24.78 mg mL^{-1} for Der p and 27.35 mg mL^{-1} for Try. **Conclusion:** An advantage of precipitation with $(NH_4)_2SO_4$ over precipitation with acetone was the possibility of gradually obtaining protein fractions, which does not happen when using the latter. The addition of 80% (v/v) acetone to the mite extracts favored total protein precipitation in the concentrations 16.42 mg mL^{-1}, 28.47 mg mL^{-1} and 13.41 mg mL^{-1}. The use of TCA in concentrations above 20% (w/v) forms peptides that are not retained in the gel under the established experimental conditions, and dilute solutions of this acid are more efficient.

Keywords: Mites, proteins, allergens, types of precipitation.

RESUMO

Introdução: As proteínas alergênicas presentes nos extratos dos ácaros de poeira, tais como *Dermatofagoides farinae* (DF), *Dermatofagoides pteronyssinus* (DP) e *Tyrophagus putrescentiae* (TP) são relevantes para estudos científicos na área de alergias e aplicação em imunoterapias. A precipitação/concentração desses extratos proteicos pode favorecer a agregação de alérgenos nos homogenatos. **Objetivo e método:** O trabalho investiga o processo de precipitação, submetendo os extratos brutos de ácaros de poeira a compostos como sulfato de amônio $(NH_4)_2SO_4$, ácido tricloroacético (ATC) e acetona. **Resultados:** Os melhores resultados foram obtidos por fracionamento com $(NH_4)_2SO_4$ em 80% (m/v) de saturação (~ 0°C), observando as marcações proteicas no gel de eletroforese. Os alérgenos principais foram identificados por immunoblot em 25 kDa (cisteína protease) para Der f 1 e Der p 1; e 25 kDa, 33 kDa (tropomyosin), 11 kDa para Tyr. Para esse percentual, os teores de proteína total foram de 12.83 mg mL^{-1} para DF; 24,78 mg mL^{-1} para DP; e 27,35 mg mL^{-1} para TP. **Conclusão:** A vantagem da precipitação com $(NH_4)_2SO_4$ frente à precipitação com acetona foi a possibilidade de gradativamente se obter frações proteicas, o que não acontece quando utilizado esse solvente. A adição de 80% (v/v) de acetona aos extratos de ácaros favoreceu a precipitação total de proteína nas concentrações 16,42 mg mL^{-1}; 28,47 mg mL^{-1}; e 13,41 mg mL^{-1}. O uso de ATC em concentrações acima de 20% (m/v) forma peptídeos que não são retidos no gel nas condições experimentais estabelecidas, sendo eficiente soluções mais diluídas desse ácido.

Descritores: Ácaros, proteínas, alérgenos, tipos de precipitação.

1. Federal University of Rio de Janeiro, Chemical Engineering Department - Rio de Janeiro, RJ, Brazil.
2. Allergenic Extracts Laboratory, Research and Development - Rio de Janeiro, RJ, Brazil.

Funding source: Laboratory of Allergen Extracts LTDA, Brazil.

Submitted: 1/14/2020, accepted: 2/20/2020.

Arq Asma Alerg Imunol. 2020;4(1):121-8.

http://dx.doi.org/10.5935/2526-5393.20200011

Introduction

The allergens found in dust mites are of great relevance for the pharmaceutical industry and are employed in many research fields in healthcare. These allergens are specific proteins that are able to cause severe mucosal inflammation, leading to rhinitis and bronchitis.[1,2] The most common house dust mites for immunotherapy with mite extracts are *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, *Blomia tropicalis* and *Tyrophagus putrescentiae*,[3-5] and therefore, these mites have greater sensibilization indexes in groups of individuals diagnosed with allergies.[6,7] The main allergens found in mite extracts are from group 1, as follows: for Der f 1: 25 kDa (cystein protease), Der f 3: 29 kDa (trypsin), Der f 10: 37 kDa (tropomyosin) and Der f 2: 15 kDa (NPC2 family); for Der p 1 : 24 kDa (cysteine protease), Der p 2: 15 kDa (NPC2 family) and Der p 3: 31 kDa (trypsin), among others, according to the *allergen.org* platform. For the *Tyrophagus putrescentiae* mite, the main allergens are Tyr p 2: 16 kDa (NPC2 family), Tyr p 3: 26 kDa (trypsin), Tyr p 10: 33 KDa (tropomyosin), Tyr p 13: 15 kDa (fatty-acid binding protein), Tyr p 28: 76 kDa (heat shock protein), Tyr p 34: 18 KDa (troponin C), Tyr p 35: 52 kDa and Tyr p 36; 14 kDa (profilin).[8]

A strategy for obtaining allergenic proteins is to expose the raw extract obtained from the mites to chemical substances capable of favoring protein isolation, pre-purification or concentration of these antigens. An interesting method that can be successfully used for protein fractionation purposes is the application of ammonium sulfate, $(NH_4)_2SO_4$, as a precipitating agent.[9] In this procedure there is interaction of water with salt ions in the medium, leading to a reduction in protein solvation by water molecules due to a change in the ionic strength of the medium. Depending on the characteristics of the protein, such as size, charge and isoelectric point (pI), the saturation of the medium may be adjusted at a given temperature to allow selective precipitation to occur. Also, some water-soluble solvents and organic acids may be used for precipitation.[10,11]

Acetone and ethanol are some of the most used solvents, allowing the solvation effect with lowering of the dielectric constant to occur and favoring protein precipitation. It is necessary, however, to conduct the procedure at low temperatures to avoid solvent denaturation. The trichloroacetic acid (TCA) forces the protein to precipitate by isolating the water around it and has the advantage of total or partial removal of high molecular weight proteins and peptides, and the precipitation rate depends on the type of protein and precipitate, as well as their respective concentrations.[12,13] Due to a high demand in areas such as vaccine research, immunotherapy and diagnostic procedures, there is a great need for the isolation and concentration of allergens found in mites from crude extracts. Thus, the present paper aimed to investigate and evaluate pre-purification and concentration methods that allow obtaining allergenic proteins from the removal of impurities present in the crude extracts of house dust mites, in order to employ these allergens in different immunochemical procedures.

Experimental section

Extract preparation

Mite cultivation and crude extract preparation were conducted as described by Mihos.[14] Dust samples were collected at households in the city of Rio de Janeiro, Brazil. Mites were isolated from 80 samples of mesh aspirated powder and identified by taxonomic reference catalogues and identification keys.[15,8] The preparation of the extracts were conducted following the procedure described by Mihos.[14]

Application of D. farinae*,* D. pteronyssinus *and* T. putrescentiae *pre-purification methods*

$(NH_4)_2SO_4$ fractioning

The crude mite extracts were sequentially fractionated with $(NH_4)_2SO_4$, as described by Scopes.[16] Fractioning was done in the ranges of 0-20% (2.82 g), 20-40% (3.02 g), 40-60% (3.25 g), 60-80% (3.50 g) and 80-100% (3.80 g) (w/v) of saturation. The percentages of added salt were determined in relation to the volume of the extract, considering a 50.0 mL sample, Scopes (1994). During precipitation, the sample remained in an ice bath at a temperature of approximately 0 °C for a period of 60-90 minutes. After this time the extract was centrifuged at 5724 xg under refrigeration at 4 °C for 20 minutes. This process was repeated for all saturation intervals. The pellets from each interval were resuspended in 5.0 mL of ultrapure water. The pre-purified extracts were stored at -20 °C for subsequent determination of protein content.

Acetone precipitation

A 50.0 mL sample of the crude extract of the study mites was added separately in ice-wrapped containers. For each extract volume were added 20%

(v/v), 40% (v/v), 60% (v/v), 80% (v/v) and 100% (v/v), respectively. Acetone was previously cooled to -20 °C. The pellet formed was centrifuged at 5724 xg at 4 °C for 30 minutes and then resuspended in 5.0 mL of ultrapure water.

TCA precipitation

The percentages of 20% (w/v), 40% (w/v), 60% (w/v), 80% (w/v) and 100% (w/v) of TCA were individually added to 50.0 mL of crude mite extract. After 30 minutes of gentle shaking the pellet formed was centrifuged at 5724 xg at 4 °C for 30 minutes and then resuspended in 5.0 mL of ultrapure water. The precipitates formed and the supernatant were stored at -20 °C for further analysis.

Precipitate characterization

Total protein content determination

The protein content of precipitates and supernatants for each pre-purification sample were determined by the Lowry method according to the literature.[17] An analytical curve was built from the standard bovine serum albumin protein (BSA, Sigma-Aldrich) in the range of 5.0 µg mL^{-1} to 100.0 µg mL^{-1}. In a test tube 1.0 mL of the BSA standard solution was added; 1.0 mL of alkaline copper reagent in 1:2:1 ratio of 0.1% (w/v) copper sulfate, sodium dodecyl sulfate (SDS), 3.2% (w/v) sodium hydroxide, respectively. After homogenization, the tubes were left to stand and 500 µL of diluted Folin-Ciocalteu reagent was added in a proportion of 1:5. The tubes were kept in the dark for 30 min. Absorbance readings were taken at 750 nm using ultraviolet-visible spectrophotometer (Spectrophotometer SP 1102, Bel photonics, Brazil).

Protein characterization by SDS-PAGE

The profile of the protein found in the precipitates and supernatants was determined by denaturing electrophoresis according to the method described by Laemmli.[18] The gel was prepared by polymerization of 30% (29:1) acrylamide/bis-acrylamide reagent (Bio-Rad). The resolving gel was prepared at 12% acrylamide/bis-acrylamide, and the stacking gel was prepared at 5.0% reagent concentration. The polymerization started after the addition of 1.0% (w/v) ammonium persulfate. Samples applied to the gel were diluted 1:1 in mercaptoethanol and bromophenol blue sample buffer solution.

Reactivity of allergenic extracts by immunoblotting

In order to evaluate the reactivity and stability of the fractions obtained from the $(NH_4)_2SO_4$ precipitation process, some samples were taken for reactivity testing against the serum of individuals diagnosed with allergies to dust mites. The fractions were selected considering the highest protein concentration obtained by the precipitation method. After electrophoretic run of the samples, the proteins separated by molecular weight were transferred to the nitrocellulose membrane with 0.45 µm pores (Bio-Rad) in a transfer module under conditions of 10 mV, 1W for 18 h at 8 °C, using as saline buffer pH electrolytic solution with pH = 7.2, consisting of 25 mM Tris-base, 192 mM glycine and 20% methanol. After the transfer, the membrane containing proteins was left in contact for 30 minutes with 5% (w/v) BSA solution to block non-membrane bound sites. After washing with TBS-T (Tris-buffered saline with Tween), the membrane was incubated in a 1:200 diluted serum pool for 40 minutes. The washing process was repeated, and the membrane was incubated in 1:2000 diluted peroxidase-conjugated anti-IgE solution. The spots related to the antigen-antibody reactivity were identified using 4-Chloro-2-nitrophenol reagent in the presence of hydrogen peroxide.

Results and Discussion

The protein content quantification of *D. farinae*, *D. pteronyssinus* and *T. putrescentiae* for the different pre-purification/concentration methods was determined according to Lowry.[17] The results are shown in Tables 1, 2 and 3.

$(NH_4)_2SO_4$ precipitation

As shown in Table 1, in the sequential fractionation precipitation method with $(NH_4)_2SO_4$, distinct molecular weight protein fractions were obtained at different times of the process. This can be observed from the protein content retained in the electrophoresis gel in the percentages of 20% in comparison with the other percentages, which was found in all fractions of mite extracts used in the study (Figure 1).

For the *D. farinae* mite extract, the salt saturation percentage at 20% (w/v) favored the precipitation of proteins smaller than 15 kDa that left the gel pores during the electrophoretic run. This finding was confirmed by the absence of proteins in the 12.5%

Table 1
Protein content (mg mL^{-1}) of dust mite extracts after $(NH_4)_2SO_4$ fractionation process, at ~0 °C.

Mite	Crude extract	0-20% (w/v)	20-40% (w/v)	40-60% (w/v)	60-80% (w/v)	80-100% (w/v)*	S
DF	4.84 (±0.05)	14.77 (±0.65)	18.00 (±0.67)	11.22 (±0.37)	12.83 (±0.49)	9.61 (±0.49)	ND
DP	4.45 (±0.05)	15.42 (±1.16)	21.87 (±0.67)	19.29 (±0.85)	24.78 (±0.19)	17.67 (±1.45)	ND
TP	6.20 (±0.00)	3.84 (±0.09)	24.74 (±0.00)	28.96 (±0.00)	27.35 (±1.30)	22.51 (±0.85)	ND

DF = *Dermatophagoides farinae*, DP = *Dermatophagoides pteronyssinus*, TP = *Tyrophagus putrescentiae*, S = supernatant, ND = not determined.

Table 2
Lowry protein content (mg mL^{-1}) for dust mite extracts after precipitation process with TCA, % in w/v

Mite	Crude extract	20% (w/v)	40% (w/v)	60% (w/v)	80% (w/v)	100% (w/v)
DF	4.45 (±0.01)	7.06 (±0.34)	10.45 (±0.56)	7.87 (±0.32)	8.03 (±0.25)	7.87 (±0.43)
S	–	17.35 (±1.61)	10.58 (±0.65)	9.93 (±1.34)	10.25 (±1.97)	8.96 (±0.19)
DP	4.84 (±0.01)	8.32 (±0.32)	6.71 (±0.99)	6.38 (±0.32)	5.09 (±0.37)	3.80 (±0.56)
S	–	ND	5.85 (±0.00)	5.15 (±0.11)	25.74 (±0.15)	17.67 (±0.21)
TP	6.20 (±0.01)	18.00 (±0.97)	12.84 (±1.16)	4.77 (±0.65)	4.13 (±0.65)	4.45 (±0.32)
S	–	17.35 (±0.34)	10.58 (±0.32)	9.93 (±0.81)	10.25 (±0.16)	8.96 (±0.09)

DF = *Dermatophagoides farinae*, DP = *Dermatophagoides pteronyssinus*, TP = *Tyrophagus putrescentiae*, S = supernatant.

Table 3
Protein content (mg mL^{-1}) for dust mite extracts after acetone precipitation process, % (w/v)

Mite	Crude extract	20% (w/v)	40% (w/v)	60% (w/v)	80% (w/v)	100% (w/v)
DF	4.45 (±0.01)	11.69 (±0.37)	14.49 (±2.60)	16.42 (±0.37)	16.42 (±0.37)	17.93 (±0.37)
DP	4.84 (±0.01)	13.63 (±0.37)	22.02 (±0.37)	26.75 (±0.37)	28.47 (±0.37)	25.46 (±0.99)
TP	6.20 (±0.00)	2.44 (±0.37)	5.67 (±0.04)	10.83 (±0.98)	13.41 (±1.34)	14.70 (±0.74)

DF = *Dermatophagoides farinae*, DP = *Dermatophagoides pteronyssinus*, TP = *Tyrophagus putrescentiae*, S = supernatant.

acrylamide/bis-acrylamide gel (Figure 1A). However, although the highest protein content was identified at 40% (w/v) saturation (18.00 mg mL^{-1}) (Table 1), it showed a low concentration of the allergenic protein of interest, cysteine protease, of 25 kDa molecular weight, which was identified in higher concentration in the 60-80% saturation fraction.

Cysteine protease has relevance in the field of immunotherapy and is a major dust mite allergen, classified in allergen group 1.[19] For the other mites the 80% (w/v) saturation percentage also showed better separation with protein contents of 24.78 mg mL^{-1} and 27.35 mg mL^{-1} for the *D. pteronyssinus* and *T. putrescentiae* mites, respectively (Table 1).

For the fractions obtained in the supernatants, after 100% (w/v) saturation, due to the low protein content, it was not possible to determine the protein levels within the detection limit proposed by the Lowry standard method.[16] In view of the results obtained in the electrophoretic run (Figure 1) the fractionation separation process was maintained in the 80% (w/v) $(NH_4)_2SO_4$ pre-purification fraction. This saturation percentage was used to evaluate the immunochemical reactivity between allergenic protein and serum IgE by western blot technique.

TCA

The fractions collected from the precipitate and supernatant by the 2,2,2-TCA precipitation method showed a similar protein content, which indicates an efficient decrease when compared to the salting-out method using $(NH_4)_2SO_4$ in the same percentages of interest (Table 2).

The poor 12.2% gel protein retention in percentages above 20% (w/v) (TCA) was observed by the electrophoresis test for the *D. farinae* mite extract (Figure 2). It can be inferred that, in percentages above 20% (w/v), and therefore a more acidic solution, peptide hydrolysates are formed and are not retained in the gel, remaining only proteins greater than 20 kDa in the polyacrylamide mesh. Also, the percentages of interest for the precipitation with TCA acid must be well below 20% (w/v) to preserve the protein structure and to obtain a good separation profile, being the target of investigation. In another study, Rajalingam et al.[10] evaluated protein precipitation (lysozyme, carbonic anhydrase and BSA) in a 0-90% (w/v) saturation range with TCA and obtained higher protein precipitation in 45% (w/v) of the acid. However, the authors reported that depending on the protein structure, percentages below 5% (w/v) are enough for the protein structure to

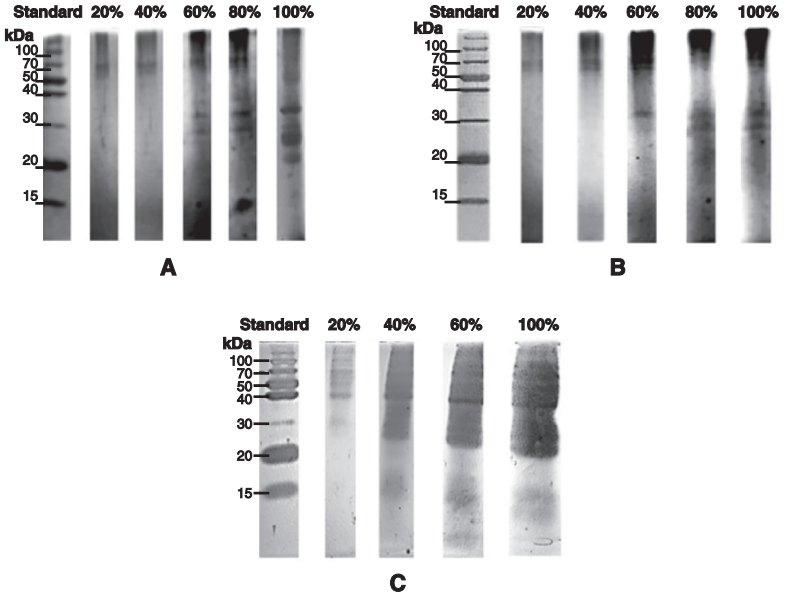


Figure 1
SDS-PAGE 12.5% under denaturing conditions for (**A**) *Dermatophagoides farinae*, (**B**) *Dermatophagoides pteronyssinus* and (**C**) *Tyrophagus putrescentiae*

unfold, exposing solvent-accessible non-polar surfaces and thus resulting in coalescence and precipitation. For the other acrylamide/bis-acrylamide gel mites they did not report electrophoretic gait.

Precipitation with acetone is a very useful method when the aim is to obtain a protein concentrate from an extract, either animal or vegetable. In order to evaluate the established working percentages of 20 to 100% of the precipitating agents in this study, the percentage concentration with solvent was evaluated using acetone for the allergenic extracts of the *D. farinae*, *D. pteronyssinus* and *T. putrescentiae* mites, as shown in Table 3.

According to the protein contents obtained (Table 3), there is an increasing trend in precipitation with the addition of acetone. The electrophoresis gel indicates that there is no selection of protein fractions, but actually full protein loading under the established conditions. These results show that the solvent method is more efficient for concentration purposes, and one strategy for isolation of these is the application of exclusion chromatography, for example.

Precipitation trends and efficiency of the methods applied for pre-purification and allergen concentrations can be better seen in the curves obtained for the mite extracts, as shown in Figure 4A, 4B and 4C.

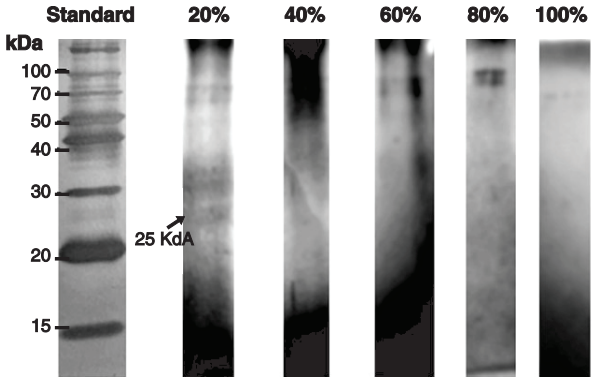


Figure 2
SDS-PAGE 12.5% under denaturing conditions for precipitation of allergic extracts for *Dermatophagoides farinae* with trichloroacetic acid (TCA)

Allergenic protein reactivity

The confirmation of allergen reactivity for protein fractions obtained in the fractionation process with 80% (w/v) of $(NH_4)_2SO_4$ salt is shown in Figure 5.

Antigen-antibody reactivity was confirmed when added in contact with serum pool of individuals diagnosed with allergy to the mites under study, containing specific IgE, which were identified at 25 kDa, for Der f 1 and Der p 1 indicating the presence

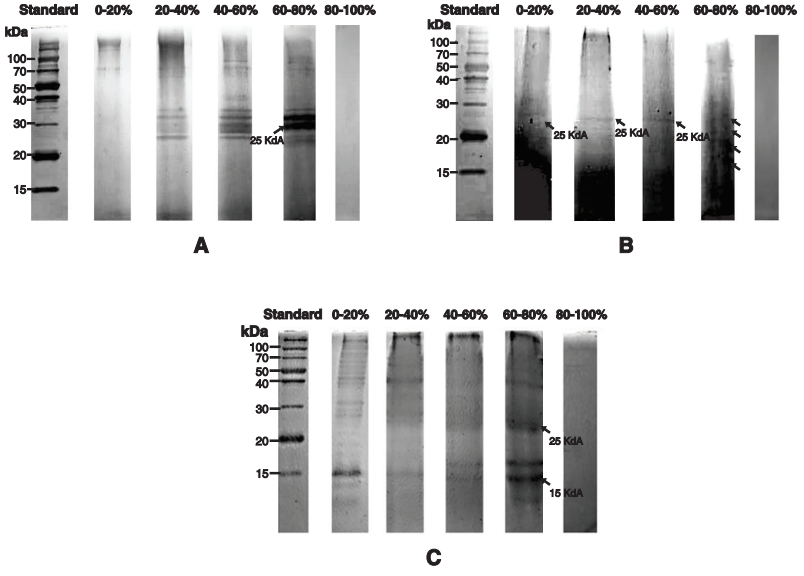
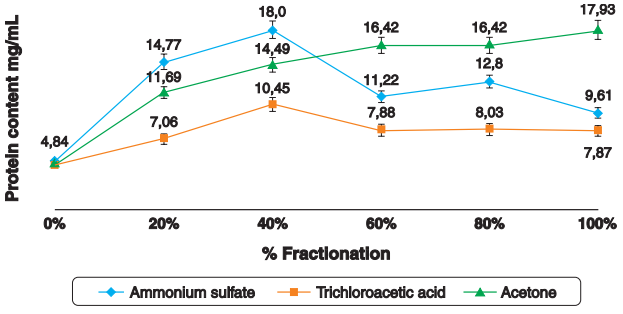
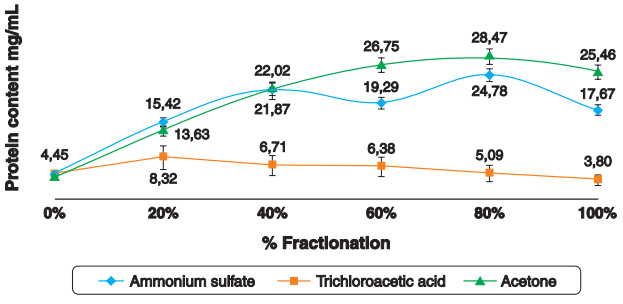


Figure 3
SDS-PAGE (12% acrylamide/bis-acrylamide) under denaturing conditions for (**A**) *Dermatophagoides farinae*, (**B**) *Dermatophagoides pteronyssinus* and (**C**) *Tyrophagus putrescentiae*

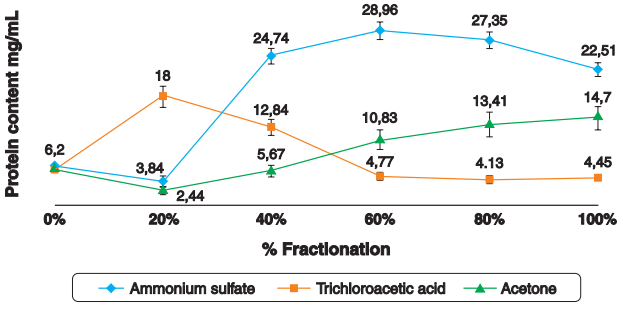
of the allergen group 1, cysteine protease. For *T. putrescentiae* mite, reactivity in the blot characterizes the allergens Try p 3, close to 26 kDa, for trypsin, and Try p 10, close to 30 kDa, for tropomyosin. Especially for *T. putrescentiae* mite, reactivity occurred for protein fractions close to 10 kDa, and this fraction was also visible in the SDS-PAGE gel (Figure 1C), below 15 kDa. For this allergen, a characterization study with proteomic analysis is necessary.



(A) *Dermatophagoides farinae*



(B) *Dermatophagoides pteronyssinus*



(C) *Tyrophagus putrescentiae*

Figure 4
Performance of the methods used for protein precipitation (mg mL^{-1}) in dust mite extracts

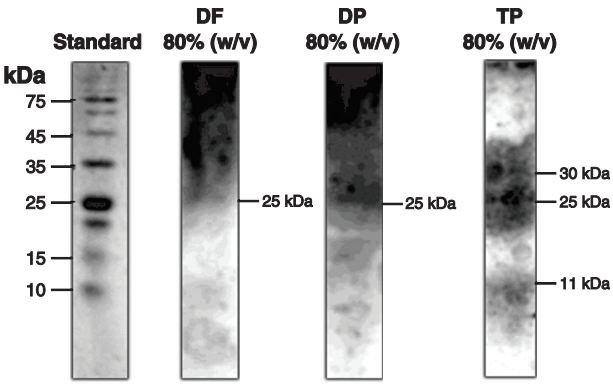


Figure 5
Immunoblotting for extracts of mites *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Tyrophagus putrescentiae* (TP) with ammonium sulfate at a saturation percentage of 80% (w/v)

Conclusion

Studies in the area of allergies and immunotherapies have great interest in purified fractions of dust mite allergens due to the low concentrations of these biomolecules in the final extraction process. Thus, the application of pre-purification and concentration techniques are well accepted for this purpose. The data presented in this paper show that the $(NH_4)_2SO_4$ fractionation process is a feasible way to obtain a more concentrated extract and that it can be used in immunodetection methods without previous salt removal, such as western blotting. The method to be applied, however, should be chosen considering the purpose of concentration and/or pre-purification.

Acknowledgment

We thank the Laboratory of Allergen Extracts LTDA, Brazil, for laboratory support.

References

1. Sun C, Liu W, Wang X, Li B, Cai J, Zou Z, et al. Associations of household dust mites (Der p 1 and Der f 1) with childhood health outcomes masked by avoidance behaviors. Build Environ. 2019;151(January):198-206.
2. Rigonato-oliveira NC, Brito AA De, Vitoretti LB, Moraes GDC, Gonçalves T, Herculano KZ, et al. Effect of Low-Level Laser Therapy (LLLT) in Pulmonary Inflammation in Asthma Induced by House Dust Mite (HDM): Dosimetry Study. Int Jounal Inflamm. 2019;2019:12.

3. Mihos F das CSS, Pereira PR, Matos AB de A, Mergh CV, Cruz MQ da. Study of protein profile and immunochemical reactivity for extracts of D. farinae, D. pteronyssinus and B. tropicalis mites in the city of Rio de Janeiro, Brazil. Arq Asma, Alerg e Imunol. 2017;1(4):379-86.
4. Herman J, Thelen N, Smargiasso N, Mailleux AC, Luxen A, Cloes M, et al. Der p 1 is the primary activator of der p 3, der p 6 and der p 9 the proteolytic allergens produced by the house dust mite Dermatophagoides pteronyssinus. Biochim Biophys Acta - Gen Subj. 2014;1840(3):1117-24.
5. Thomas WR, Smith W-A, Hales BJ. The allergenic specificities of the house dust mite. Chang Gung Med J. 2004;27(8):563-9.
6. Boonpiyathad T, van de Veen W, Wirz O, Sokolowska M, Rückert B, Tan G, et al. Role of Der p 1-specific B cells in immune tolerance during 2 years of house dust mite-specific immunotherapy. J Allergy Clin Immunol. 2019 Mar;143(3):1077-1086.e10.
7. Malet A, Azpeitia A, Gutiérrez D, Moreno F, San Miguel Moncín M del M, Cumplido JA, et al. Comprehensive study of patients' compliance with sublingual immunotherapy in house dust mite perennial allergic rhinitis. Adv Ther. 2016 Jul;33(7):1199-214.
8. Colloff MJ. Dust mites. Netherlands: Springer; 2009. p. 1-583.
9. Turkheimer E, Waldron M. The Role and Efficiency of Ammonium Sulphate Precipitation in Purification Process of Papain Crude Extract. Psychol Bull. 2019;126(1):21.
10. Rajalingam D, Loftis C, Xu JJ, Kumar TKS. Trichloroacetic acid-induced protein precipitation involves the reversible association of a stable partially structured intermediate. Protein Sci. 2009;18(5):980-93.
11. Fic E, Kedracka-Krok S, Jankowska U, Pirog A, Dziedzicka-Wasylewska M. Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis. Electrophoresis. 2010;31(21):3573-9.
12. Greenberg NA, Shipe WF. Comparison of the abilities of trichloroacetic, picric, sulfosalicylic, and tungstic acids to precipitate protein hydrolysates and proteins. Journal. J Food Sci. 1979;44(6):735-7.
13. Yvon MCC, Pélissier JP. Solubility of peptides in trichloroacetic acid (TCA) solu tions. Int J Pept Protein Res Haguenau. 1989;34(1):166-76.
14. Mihos F, Paiva V, Pereira P, Matos A, Cruz M. Immunological analysis of allergenic cross-reactivity between Cheyletus malaccensis and Dermatophagoides farinae , Dermatophagoides pteronyssinus and Blomia tropicalis. Arq Asma Alerg Imunol. 2018;2(2):247-52.
15. Flechtmann CHW. Elements of Acarology. 1st ed. São Paulo: Nobel; 1975. p. 9-339.
16. Fedatto LM. Caracterização de proteases extracelulares produzidas por Xylella fastidiosa de citros e videira [thesis]. Piracicaba (SP): ESALQ/CENA-USP; 2004.
17. Lowry OH, Rosebrough J, Lewis A, Randal R. Medición de proteínas con el reactivo de fenol Folin. J Biol Chem. 1951;193(1):265-75.
18. Laemmli UK. Cleavage of Structural Proteins during the Asssembly of the Head of Bacteriophage T4. Nature. 1970 Aug 15;227(5259):680-5.

No conflicts of interest were declared associated with the publication of this article.

Corresponding author:
Francisca das Chagas Sobral Silva
E-mail: franciskasobral@gmail.com