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Research Article

Development of a glutaraldehyde-polymerized *Blomia tropicalis* extract (allergoid) for Allergen Immunotherapy

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Abstract

A curative treatment for hypersensitivity to Blomia tropicalis is yet far from being ideally achieved; however, desensitization strategies conducted with unmodified natural extracts and chemically modified allergens (allergoids) are promising therapeutics. This study aims to evaluate and compare methodologies to culture HDM, extract their proteins, and produce allergoids by glutaraldehyde polymerization. We compared the growth of Blomia tropicalis in two different culture media. Medium A was prepared with yeast extract and rodent ration. Medium B was prepared with yeast extract and Tetramin®. We compared the extraction of the proteins performed with two different buffers: Phosphate Buffered Saline (PBS) and Borate Buffer (BBS). After extraction, the distribution of proteins was demonstrated by SDS-PAGE, and the native extract was compared with its glutaraldehyde-polymerized allergoid. Both cultures were inoculated with approximately 2,000 mites/g of culture. The population peak was reached after 105 days in medium A (52,000 mites/gram of culture) and 90 days in medium B (71,000 mites/gram). Therefore, faster population growth and a higher population density are observed in medium B. SDS-PAGE showed that the allergens' low molecular bands disappeared after polymerization, obtaining polymers with a molecular weight greater than 150 kDa. For *B. tropicalis*, the yeast extract enriched with Tetramin® as a culture medium presented better results for growing; however, both media and extraction buffers enabled the production of a native protein extract and, from this, the production of a polymerized allergoid.

1. Introduction

Environmental allergens mainly contribute to immune dysregulation by promoting oxidative stress, epithelial barrier dysfunction, Th2-skewed inflammation, and exacerbating allergic diseases [1]. The allergenicity of the Blomia genus and its cross-reactivity with *Dermatophagoides* species were described in Japan in the late 1960s [2]. The species Blomia tropicalis was described in the early 1970s [3]. After this, several authors reported the association of *B. tropicalis* with respiratory allergic symptoms in tropical regions [4–8]. Further, some reports indicated that food contamination with *Blomia tropicalis* produced urticaria, anaphylaxis, atopic dermatitis, and hypersensitivity to non-steroidal anti-inflammatories [9–11].

The first attempts to classify house dust mite (HDM) allergens were performed with *D. pteronyssinus*. The subsequent classifications of HDM-related allergens (such as *B. tropicalis*) followed the *D. pteronyssinus* cataloguing [12]. Sharing amino acid sequence homology with

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the *D. pteronyssinus* allergen Der p 5, the Blo t 5 was soon considered the major allergen from *B. tropicalis* [13]. Blo t 5 has a triple-helical bundle fold with a slow conformational exchange on its epitope surface [14].

In vitro comparisons of IgE responses to *B. tropicalis*, rBlo t 5, and *D. pteronyssinus* rDer p 5 showed that *B. tropicalis* has unique allergens that may represent an independent cause of sensitization [15]. Blo t 5 does not exhibit significant levels of cross-reactivity with Der p 5, indicating that specific extracts are necessary to diagnose allergies and prescribe distinct therapeutic desensitization against these mites [16].

The Allergen Nomenclature Sub-Committee of the World Health Organization and International Union of Immunological Societies (WHO/IUIS) has recognized so far twenty-six allergens weighing from 7 to 110 KDa, identified from *B. tropicalis* [17, 18].

Allergen immunotherapy (AIT) is the only curative approach to treat allergic diseases [19]. The main limitation of AIT is its intrinsic allergenicity derived from allergens' conformational epitopes, while its therapeutic (immunogenic) activity derives from its linear epitopes [20]. Allergoids are modified immunotherapeutic extracts designed with reduced allergenicity and sufficient immunogenic activity for therapeutic desensitization of allergic patients [21–24].

Polymerized allergen extracts are easily produced with the help of aldehydes (such as formaldehyde and glutaraldehyde), or by biocatalysis, originating in the conceptualization of allergoids [25–27].

This work aims to study more efficient ways to culture *B. tropicalis* on an industrial scale, comparing two different culture media and two different extraction buffers in order to optimize the production of diagnostic and therapeutic native allergenic extracts as well as the production of polymerized allergoids from these extracts.

2. Materials and methods

2.1. Original mite culture medium

The cultures of *B. tropicalis* were kindly provided by Prof. Dr. Jorge Martinez Quesada from the Faculty of Pharmacy- University of the Basque Country – Spain. Some of the characteristics that we looked for the microscopic characterization of *Blomia tropicalis* were: very long and very serrated setae; absent coxal apodemes III and IV; very serrated legs with setae, very thin tarsi; absence of a propodosomal shield; presence of two pairs of trapezoidal setae in the place where the shield would be and sinuous copulatory tube on the posterior part of the mite's body see Figure 1 [28].

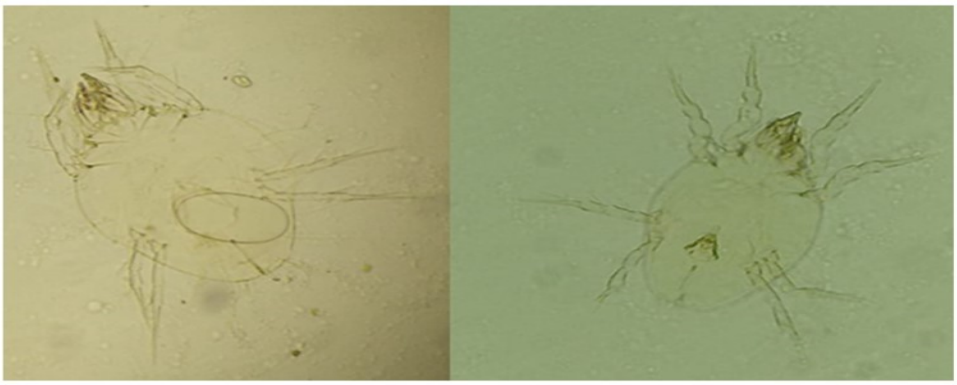

Figure 1: Blomia tropicalis female with an egg (left) and male (right). Pictures taken with the 10x magnification objective lens of the phase contrast microscope

Blomia tropicalis cultures were prepared and brought to Brazil in triplicate. The culture medium of yeast extract (Vigor®, Santiveri, Barcelona) and commercial mouse food (A.04 Panlab®, Barcelona) in a 1:1 ratio was mixed and ground in a Thermomix® until a fine, homogenized powder was obtained. The culture medium was poured into a previously sterilized Erlenmeyer flask.

Before inoculating the mites, the culture medium was placed in 50 mL ThermoFisher® cell culture flasks (15 g per flask) in an aseptic environment, and these flasks were placed in an airtight container containing a saturated solution of distilled water and sodium chloride for at least 24 hours to humidify the culture medium. Approximately 10 small spatulas (weighing on average 0.0295 g each) were inoculated from the culture at the optimum point for inoculation (exponential growth) according to the growth curve study, that is, the number of mites/g of culture was at the maximum that could be reached for that culture used for inoculation. To arrive at this number of inoculated spatulas, simple calculations were performed to ensure that the number of mites inoculated in each vial was approximately 3,600 mites/g of culture. To stabilize cultures, cultures were inoculated approximately one month before the trip to Brazil inside airtight bags containing cotton soaked in the saturated NaCl solution, thus maintaining humidity during the flight.

2.2. Preparation of the culture medium – Bio Allergy

The ingredients used to prepare the culture media are yeast extract (Synth) with rodent ration (Medium A) and yeast extract (Synth) with Tetramin® (Medium B). The ingredients were weighed on the Thermomix's built-in scale (Vorwerk, TM6®). They were then crushed using the device's primary function for 30 seconds at maximum speed (10), and this step was repeated three times until a fine, homogenized powder was obtained.

After the grinding step in the Thermomix®, all culture media were sieved through a 60 mesh sieve (250 μm opening) to ensure standardization and granulometry of the medium. The sieved culture medium was then weighed (10 g) and placed in Erlenmeyer flasks with a cotton plug and gauze to be transferred to a sterilization and drying oven (Ethik Technology, 400D®), previously heated, where it

remained for 1 hour at 110 °C. After cooling, with the help of a plastic funnel also previously sterilized, the contents of the Erlenmeyer flasks were poured into 50 mL cell culture flasks, in a unidirectional flow, previously treated with ultraviolet light for 15 minutes, to prevent contamination by microorganisms and other species of mites. The 50 mL culture flasks containing only the culture medium were kept in airtight boxes with a saturated sodium chloride solution and distilled water for at least 48 hours to humidify them before inoculating the mites.

To standardize that all new inoculated crops would start with a proportion of 8,000 mites/g of crop, the initial culture used for inoculation would first undergo a count to estimate the number of mites/g present. To perform the counts in this study, a small sample from each culture (between 50 and 100 mg) was removed and weighed on an analytical balance, and the result was recorded. The sample was placed in a Petri dish divided into quadrants below the stereomicroscope, and with the aid of a needle or fine-bristled brush, the live mites were counted and removed from the plate. The mites considered alive for all counts performed in this study were mobile or moved when touched. This estimate was expressed in grams of the initial culture, and precisely this quantity was removed from the cell culture flask, within the unidirectional flow and with the aid of a spatula, and weighed on an analytical balance previously sanitized with 70% alcohol to achieve the proportion of 8,000 mites/g of crop. This portion, containing many live mites in all stages of development, bodies of dead mites, feces, metabolites of the mites and their microenvironment, among other substances, was then inoculated into a new vial containing only new culture medium, free of metabolites and rich in nutrients, ready for inoculation, as described above. After this step, the vials were cleaned on the mouth and externally with 70% alcohol and sealed with Parafilm® M (Bemis) between the vial and the lid to prevent the mites from escaping through the gaps in the vial closure. A ”vent” type lid with a 0.22 μm hydrophobic filter allowed the culture to exchange gas with the outside air and maintain the humidity and sterility conditions inside the flask. The new crops were labeled with the matrix code, and their information for future tracking was described in a crop control spreadsheet. After being labeled, they were sent to the biochemical oxygen demand thermal chamber (Solid Steel, SSBODu®) at 25 °C $\pm$ 2 °C and 75% RU $\pm$ 5% RU, which contained only matrices of *B. tropicalis*.

2.3. Production of native allergenic extract

The production process of the native allergen extract on a laboratory scale continued after the optimum point of the growth curve was confirmed, interrupting the cultivation by freezing the mite culture samples in a freezer at -20 °C for 24 hours. The culture containing mites (adults, nymphs, larvae, feces, and eggs) and the culture medium were weighed to extract the allergens.

Two types of buffers for protein solubilization were tested, the Phosphate Buffered Saline (PBS with pH 7.2; composition per Liter: Sodium Chloride (NaCl) – 81.9 g; Potassium Chloride (KCl) – 1.87 g; Dibasic Sodium Phosphate (Anhydrous Na_2HPO_4) – 14.19 g; Monobasic Potassium Phosphate (KH_2PO_4) - 2.38 g and the BBS (Borate Buffer) pH 8.4; composition per Liter: Boric Acid (H_3BO_3) – 61.83 g; Sodium Hydroxide (NaOH) – 10 g.

The extraction was performed at a rate of 10 mL of buffer per gram of raw material (10%), using gentle magnetic stirring (1,000 rpm) for 4 hours at 4 °C. After this process, the material was centrifuged at 5,000 rpm (3,020g for 30 minutes, and the supernatant was preserved. The sediment was resuspended under the same conditions as in the first step and kept under stirring for 2 hours. The centrifugation was then repeated, and the supernatants were mixed.

The final extract obtained was dialyzed against buffer in order to eliminate low molecular weight proteins ($< 5,000$ Da) for 24 hours, with three buffer changes and a ratio of 1:50, content to be dialyzed/buffer. The process was continued by centrifugation at 10,000 rpm (12,100g for 30 minutes at 4 °C).

2.4. Protein extraction

An 800 mg of whole culture (mites and culture medium) was weighed, so each sample had 100 mg of culture for extraction. The samples underwent the grinding process (reduction in the size of mite bodies and feces, as well as the culture medium) to improve the exposure of allergens to the extraction buffer. Next, they were subjected to the defatting process (with ethyl ether), which favors the loss of non-protein molecules. The sonication disruption process was added to provide an additional step before extraction, which could improve protein exposure and contribute to the solubilization of allergens.

All samples underwent extraction, with the only difference being the type of buffer used (Borate Buffer – BBS or Phosphate Buffered Saline – PBS), and were conducted at 4 °C.

2.5. Determination of molecular masses by reducing SDS-PAGE

SDS-PAGE analyzed samples under reducing conditions using a Mini Protean Tetra Cell apparatus (Bio-Rad, CA, USA) and 4%T/15%C gel gradients. [29, 30] Glutaraldehyde-polymerized and native *B. tropicalis* extract samples were prepared in the SDS buffer to achieve a final concentration of 5μg/μL and then heated for 5 min at 100 °C. An aliquot of 5 μL (25 μ g) was applied to each lane. A 6-180 kDa molecular mass pre-stained protein ladder (BenchMarkTM® acquired from Invitrogen) was used to identify approximate molecular weights. The applied current was 10 mA for 20 minutes until the sample entered the resolving gel and 30 mA until the end of the run. The polyacrylamide gels were stained with Coomassie Blue Colloidal, leaving them stained overnight at room temperature. They were then decolorized with a methanol/acetic acid/distilled water solution until the standard and sample bands could be seen.

2.6. Chemical Polymerization

The polymerization reaction was conducted by adding the glutaraldehyde solution (25%) to the solution containing the depigmented *B. tropicalis* extract. The polymerization reaction was conducted for six hours at 4 °C under continuous stirring. The reaction was interrupted with glycine. To verify the success of the polymerization step, quality control was performed using the SDS-PAGE methodology, comparing the pre-polymerization gel with the post-polymerization gel. For comparison between native and polymerized extracts, the same extraction batch was used in parallel to avoid variations inherent to the biotechnological process.

3. Results

3.1. Population growth dynamics

Cultivation experiments of *B. tropicalis* were conducted at the Bio Allergy - Developmental Laboratory for Research and Innovation in Allergy. For this study, 50 mL culture flasks containing 10 g of previously sterilized and humidified culture medium were used for each diet evaluated. Biweekly counts were performed, and the medium was kept until it was used up. *B. tropicalis* was cultivated in medium A and medium B. Both cultures were inoculated with approximately 2,000 mites/g of culture. The population peak was reached after 105 days in medium A (52,000 mites/gram of culture) and 90 days in medium B (71,000 mites/gram). Therefore, faster population growth and a higher population density are observed in medium B. Therefore, for *B. tropicalis*, medium B presented better results under the conditions tested in the experiment. Tables 1 and 2 show the quantification results of *B. tropicalis* in medium A and medium B, respectively. Figures 2 and 3 show the growth curve of the *Blomia tropicalis* in medium A and medium B, respectively.

Table 1: Counts of mites *(Blomia tropicalis)* in three crops carried out in Medium A with the Arithmetic Mean and Standard Deviation

Medium A Days	Culture 1 Mites/g	Culture 2 Mites/g	Culture 3 Mites/g	Mean Mites/g	SD Mites/g
0	1,578	,885	2,217	1,893	319
14	10,808	11,100	12,343	11,417	815
25	14,134	15.00	17,854	15,663	1,947
40	20,543	19,007	20,888	20,146	1,001
45	29,890	28,75	30,543	29,728	907
60	32,400	30,212	2,876	31,829	1,421
75	35,708	34,569	36,008	35,428	759
90	51,344	49,725	51,900	50,989	1,130
105	52,582	50,572	53,122	52,092	1,344
120	29,879	25,347	20,988	25,404	4,446
135	10,344	9,866	7,987	9,399	1,246
150	0	0	0	0	0

Table 2: Counts of mites *(Blomia tropicalis)*in three crops carried out in Medium B with the Arithmetic Mean and Standard Deviation

Medium B Days	Culture 1 Mites/g	Culture 2 Mites/g	Culture 3 Mites/g	Mean Mites/g	SD Mites/g
0	2,034	1,248	1,876	1876	416
14	13,988	12,546	14,122	13,988	874
25	17,843	18,766	20,320	18,766	1,252
40	23,987	24,988	26,988	24,988	1,528
45	37,876	36,122	39,122	37,876	1,507
60	43,100	41,234	43,584	43100	1,241
75	61,432	58,900	60,012	60,012	1,269
90	70,877	69,340	71,845	70,877	1,263
105	34,122	35,654	40,644	35,654	3,410
120	18,981	12,298	16,743	16,743	3,402
135	980	490	1032	980	299
150	0	0	0	0	0

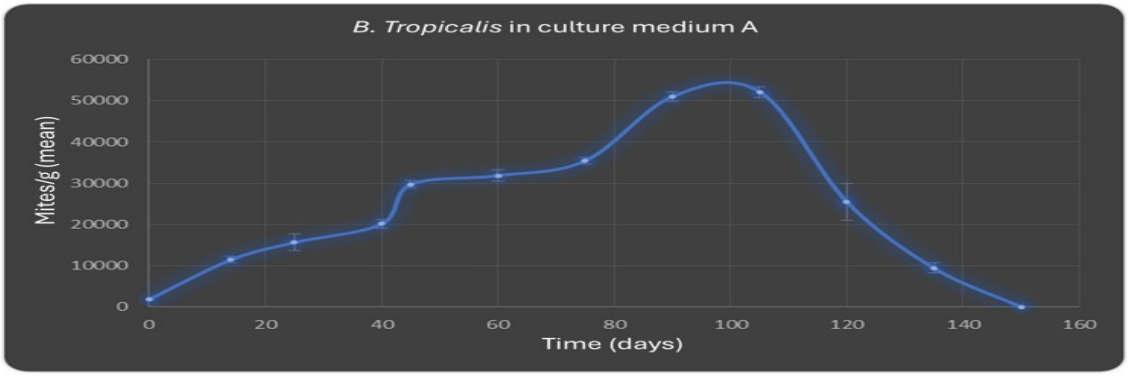

Figure 2: Growth curve (arithmetic mean) of three crops of *B. tropicalis* cultured in Medium A

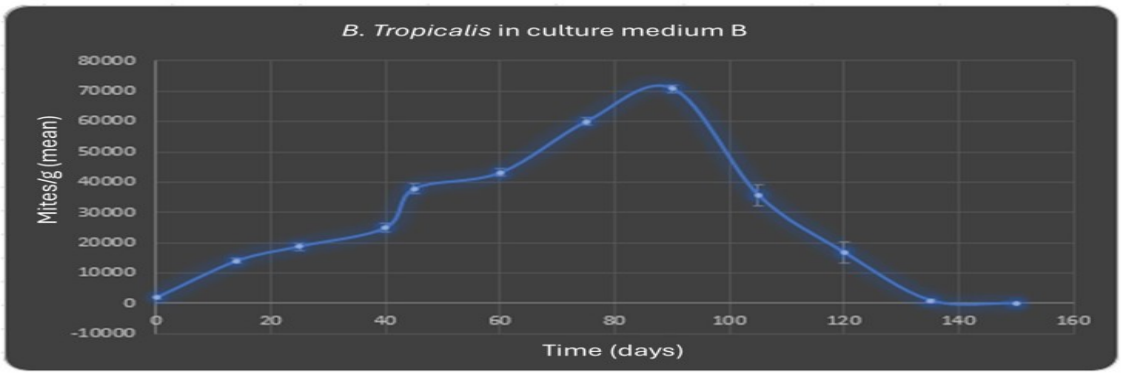

Figure 3: Growth curve (arithmetic mean) of three crops of *B. tropicalis* cultured in Medium B

3.2. Glutaraldehyde polymerization – SDS-PAGE

Four extract samples of *B. tropicalis* were subjected to glutaraldehyde polymerization.
The four native extracts and their glutaraldehyde-polymerized counterparts were put side by side in the running SDS-PAGE (see Figure 4).
The polymerized extracts were indicated by the corresponding number of the native extract as described:
Sample A: *B. tropicalis* cultured in medium A, extracted with PBS;
Sample Ap: Preparation A after glutaraldehyde polymerization
Sample B: *B. tropicalis* cultured in medium A extracted with BBS;
Sample Bp: Preparation B after glutaraldehyde polymerization
Sample C: *B. tropicalis* cultured in medium B extracted with BBS;
Sample Cp: Preparation C after glutaraldehyde polymerization
Sample D: *B. tropicalis* cultured in medium B extracted with PBS;
Sample Dp: Preparation D after glutaraldehyde polymerization

In the aforementioned analyses, it is observed that when comparing the native extract, that is, allergens without any predictable chemical modifications, with the polymerized extract, we notice that in the native extract, it is possible to visualize bands that characterize several allergens of *B. tropicalis*. As expected, after the polymerization process, these low molecular bands disappeared, since the molecular weight of these proteins increased considerably, obtaining polymers with a molecular weight greater than 150 kDa.

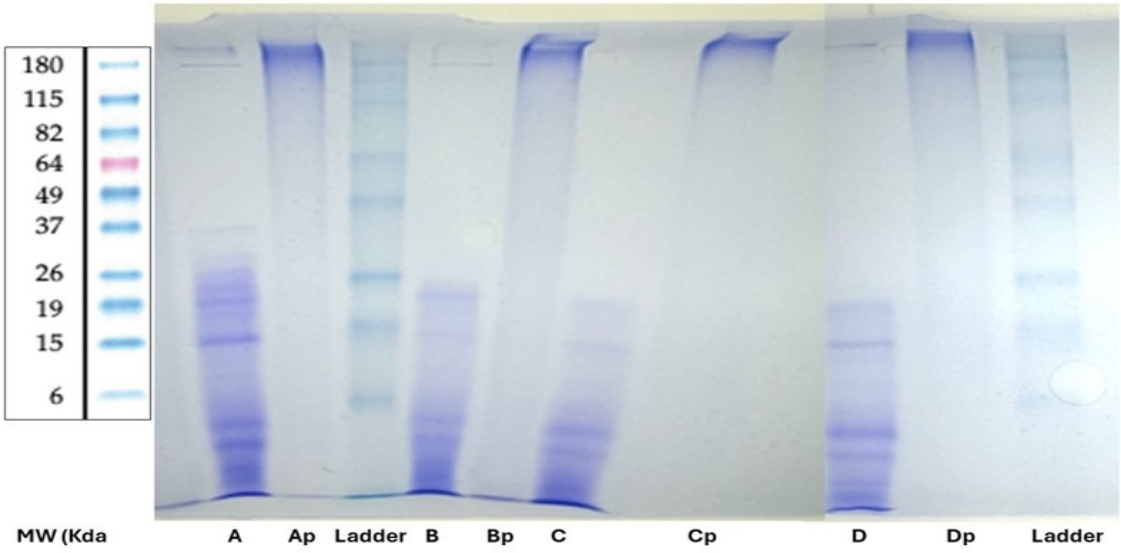

Figure 4: Figure 4 SDS-PAGE with eight preparations of *Blomia tropicalis*.

A: Medium A extracted with PBS; Ap: Preparation A after glutaraldehyde polymerization
B: Medium A extracted with BBS; Bp: Preparation B after glutaraldehyde polymerization
C: Medium B extracted with BBS; Cp: Preparation C after glutaraldehyde polymerization
D: Medium B extracted with PBS; Dp: Preparation D after glutaraldehyde polymerization

3.3. Discussion

House Dust mites are the primary respiratory allergen affecting allergic patients, producing debilitating conditions such as allergic rhinitis, allergic bronchitis, allergic sinusitis, allergic pharyngitis, and atopic dermatitis [31–35]. Immunopharmacology of allergic diseases is based on desensitization strategies using native and modified allergens to obtain tolerance [36, 37].

Technical research to obtain industrial doses of mites' allergens is crucial to providing supportive extracts for diagnostic and medical therapeutic procedures destined for allergen-specific, group-specific, native, and/or allergoid desensitization [38, 39].

Medium B (yeast extract enriched with Tetramin® presented better results, allowing a faster growth of *B. tropicalis* and a higher density population, probably due to the Tetramin® supplementation with a high protein content (47g/100g). Both buffers (PPS and BBS allowed a similar protein extraction as demonstrated by the similar SDS-PAGE runs Figure 4. The SDS-PAGE also demonstrated a similar polymerization in the four samples cultured in Medium A or B or extracted in PBS or BBS.

The primary effect of aldehydes in proteins is to covalently cross-link the primary amine group on the lysine side chains [40, 41]. The glutaraldehyde polymerized extracts reduce allergenicity and increase the regulatory-to-effector T cell ratio [42, 43]. Besides chemical polymerization, biological polymerization may be achieved through enzymes such as the transglutaminases [44–46].

Polymerization produces large molecules $(10^5 to 10^6 Da)$, preventing their direct absorption by the mucosal membranes (and systemic allergic reactions) [47, 48]. This particularity turns polymerized allergoids attractive for sublingual use since the oral mucosa precedes the internalization of large proteins by phagocytosis/pinocytosis of great antigens by sampling performed by the Dendritic Cells [49].

4. Conclusion

In conclusion, for *B. tropicalis*, the yeast extract enriched with Tetramin® as a culture medium presented better results for growing, enabling the production of a native protein extract. Phosphate-based and Borate-based buffers were similarly convenient for extracting allergenic proteins, allowing the production of the native extract and the polymerized allergoid.

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