

Stability of *Pityrosporum ovale* allergens during storage

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Summary

Background *Pityrosporum ovale* is a common saprophyte on the skin capable to induce IgE antibody production in atopic dermatitis. Generally IgE response to *P. ovale* has been established with skin-prick test, but the stability of *P. ovale* skin test allergens is not known.

Objective and methods In this study we analysed the stability of identified allergens of *P. ovale* in skin test solutions during storage. The stability was checked with immunoblotting and densitometry after different time periods and at various temperatures.

Results Coomassie Brilliant blue staining clearly showed, that proteins were preserved poorly at +20 °C in 50% glycerol. Even extracts stored at +4 °C during 6 and 12 months in 50% glycerol were degraded. Only extract stored at +4 °C for 1 month in 50% glycerol was quite well preserved. According to densitometry results the 9 kDa band was the most stable main protein allergen. The 20 kDa and especially the 96 kDa bands were far more labile. The stability of low molecular allergens was in general better than the stability of high molecular weight allergens.

Conclusions The stability of *P. ovale* extracts stored for more than 1 month was poor even when kept at +4 °C temperature. Reliability of negative skin prick results should be questioned when older extracts are used. In skin test negative cases, the sensitization should be evaluated also with the measurement of specific serum IgE antibodies to *P. ovale*.

Keywords: allergen, *Pityrosporum ovale*, stability, skin-prick test, yeast

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Introduction

Stability of skin test allergens used for clinical diagnostic work vary a lot depending on the time period, temperature and preservatives used in the storing of the allergens [1–8]. For proper diagnostic work it is essential to know which allergens will be present in the solutions in different storage conditions. According to previous reports, allergen solutions such as extracts of wheat, rye and barley retained their allergenic activity well, even after storage at +4 °C for 21 months and at room temperature for 10 months [4]. The *Alternaria tenuis* extracts were likewise remarkably stable at different temperatures (–20, +4 and +24 °C) over a 12-month period [5]. Cocksfoot pollen extracts and *Dermatophagoides pteronyssinus* extracts behaved differently when

stored, pollen extract lost its activity in 6 months and the mite extract in 2 months [6]. Yeast allergens of *Candida albicans* (*C. albicans*) and *Saccharomyces cerevisiae* (*S. cerevisiae*) were proven to be quite labile in skin test solutions [7,8]. Addition of 50% glycerol in the test solutions has improved the stability of several allergens and it is generally regarded as a good stabilizer [3,7,8].

Pityrosporum ovale (*P. ovale*) is a common saprophyte on the skin. In patients with atopic dermatitis (AD), especially with head, neck and shoulder distributions of the dermatitis, saprophytic yeast growth contributes to exacerbations of dermatitis also inducing an IgE antibody response against the yeast antigens [9–11]. The IgE binding allergens of *P. ovale* have been characterized in several studies using an immunoblotting method. According to our earlier results in immunoblotting, 39 different IgE stained bands could be visualized, the 9 kDa, 20 kDa, 96 kDa regions and mannan, a polysaccharide allergen, being the most important [12].

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Table 1. Different storage conditions of the allergen extracts

Extract	Storage time	Temperature
Non-glycerol	0 days (fresh)	Room temperature
50% glycerol	0 days (fresh)	Room temperature
50% glycerol	1 month	+4°C
50% glycerol	6 months	+4°C
50% glycerol	12 months	+4°C
50% glycerol	1 month	+20°C
50% glycerol	6 months	+20°C
50% glycerol	12 months	+20°C
50% glycerol	12 months	+37°C
non-glycerol	12 months	+4°C
non-glycerol	12 months	+20°C

Skin-prick tests (SPT) are widely used to demonstrate specific IgE antibodies also against *P. ovale*. However, the stability of the *P. ovale* allergens in skin test solutions has not been established. Therefore we analysed in this study the stability of identified allergens of *P. ovale* during storage at various temperatures and after different time periods. The stability was checked by immunoblotting and densitometry.

Materials and methods

Patients

A serum pool of six atopic dermatitis patients, positive in *P. orbiculare* RAST and IgE-immunoblotting, was designed to detect the 9, 20, 25, 27, 40, 42, 96 kDa bands and mannan, thus including all important allergens of *P. ovale*. Serum total IgE value of the patients sera varied from 870 kU/L to 3832 kU/L.

Preparation of *P. ovale* extracts

P. ovale strain (CBS 7854) used in the assays was isolated at the Department of Dermatology by the mycological laboratory of the University Central Hospital of Turku from the scalp of a patient with seborrhoeic dermatitis and later typed and deposited in CBS (Centraalbureau Voor Schimmelcultuur, Delft, Netherlands). The yeast was grown on agar plates on medium containing reduced-fat cream supplementum according to Leeming and Notman [13]. The crude cytoplasmic extract was prepared with disruption of the cells in a X-press chamber (AB Biox, Goteborg, Sweden) as previously reported [12,14]. The final diluents were prepared by reconstituting the lyophilized antigen stored at -20°C with ALK-Diluent (0.3 mg human serum albumin in 1 mL saline, ALK, Hoersholm, Denmark) without glycerol or with 50% glycerol (Sigma, St. Louis, MO, USA). The concentration of *P. ovale* extracts were 20 mg/mL of

which 76% contained protein measured by Bichinonic acid assay (Pierce, Rockford, Illinois, USA). The extracts were filtered through filters with 0.2 µm pore size (Acrodisc PF, Gelman Sciences, Michigan). Extracts with 50% glycerol were stored for 1 month, 6 months and 1 year at +4°C and +20°C and for 1 year at +37°C. Extracts without glycerol were stored for 1 year at +4°C and +20°C. (Table 1).

SDS-PAGE and electrophoretic blotting

The *P. ovale* extracts and molecular weight marker (Rainbow coloured protein, molecular weight marker, Amersham, International PLC) were separated by SDS-PAGE in a 5–17.5% gradient gel over night according to Laemmli [15]. Twenty microlitres of each extract was diluted with equal amount of Tris buffer, adjusted to pH 6.8, containing 2% (w/v) SDS and 10% (w/v) glycerol, and incubated for 5 min at +100°C without reduction. Separated antigens were then transferred to nitrocellulose sheets (Schleicher & Schuell, Dassel, Germany) in a Transphor apparatus in 1.0 A and 1.5 h (Hoefer, San Francisco, USA) according to Towbin *et al* [16]. The protein bands on the gel were stained with Coomassie brilliant blue.

IgE antibody-detection

The specific IgE-binding of the allergens on nitrocellulose was identified as described in detail [17]. In brief, nitrocellulose sheets were first incubated in PBS-Tween 20 0.5% for 1 h to block the unoccupied binding sites and thereafter over night at 20°C with serum pool. After three washes with PBS-Tween 20 0.5%, ¹²⁵I-labelled anti-IgE antibodies (Pharmacia CAP Systems, RAST RIA, Kabi Pharmacia Diagnostics, Uppsala, Sweden) diluted 1:10 in PBS-Tween 20 0.5%, were added and incubated for 4 h at 20°C. The ¹²⁵I-labelled bands were visualized on a Trimax X-ray film in a cassette with intensifying screens (Trimax XM, 3 m, Italy) after storage at -70°C prior to developing.

Image analysis

The staining intensity of each band was measured from X-ray film by an image analysis (MCID Image Analysis, Imaging Research Inc.). Scanning was done by measuring the maximum peak. The density values were presented in relative optical density (ROD).

Results

After storage the extracts were golden brown in colour, except two; one extract not containing glycerol and stored for 1 year at +20°C, that was dark brown and the other stored for 1 year at +37°C, and containing 50% glycerol,

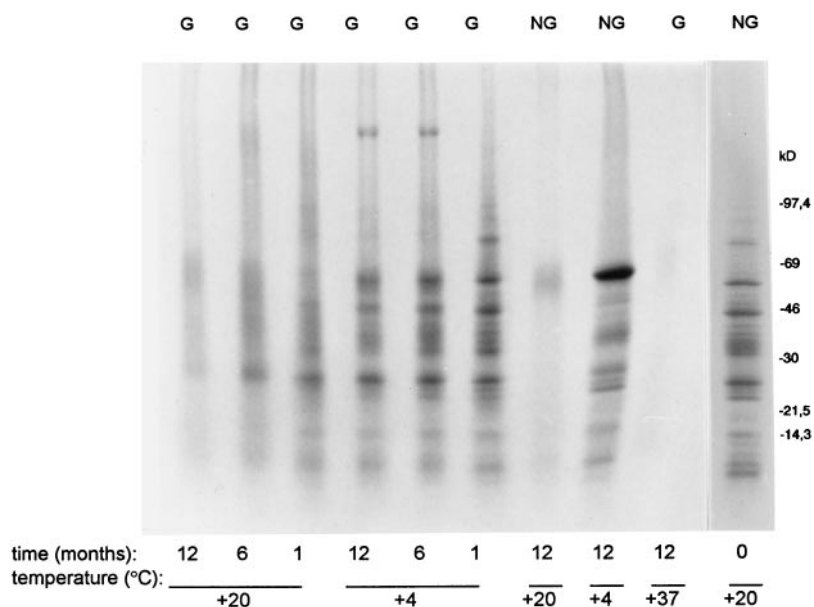


Fig. 1. Coomassie brilliant blue stained gels of *P. ovale* extracts. G is 50% glycerol containing extract and NG is nonglycerol extract.

that was medium brown. All the extracts were free of precipitates after storage.

In Fig. 1 Coomassie Brilliant blue staining shows that proteins were poorly preserved at +20°C with 50% glycerol. Even extracts stored at +4°C for 6 and 12 months in 50% glycerol were degraded, and new high molecular weight protein bands around 180 kDa had appeared in extracts. Only the extract stored at +4°C in 50% glycerol for 1 month was quite well preserved. However, the extract stored for 1 year at +4°C without glycerol was rather well preserved, but here also a protein band of degradation products around 60 kDa was seen. Extracts stored for 1

year at room temperature without glycerol and at +37°C with 50% glycerol had been degraded practically completely.

IgE immunoblotting (Fig. 2) confirmed that storage at +4°C in 50% glycerol was superior compared with that at +20°C. There were no significant differences between IgE-staining of the individual bands of freshly diluted freeze-dried extracts with or without glycerol and with or without sterile filtering.

When the extract containing 50% glycerol was stored for one month at +20°C only the 9 kDa and 20 kDa bands were clearly detected. After 6 months at +20°C in 50% glycerol the 9 kDa band was hardly seen and the other bands had

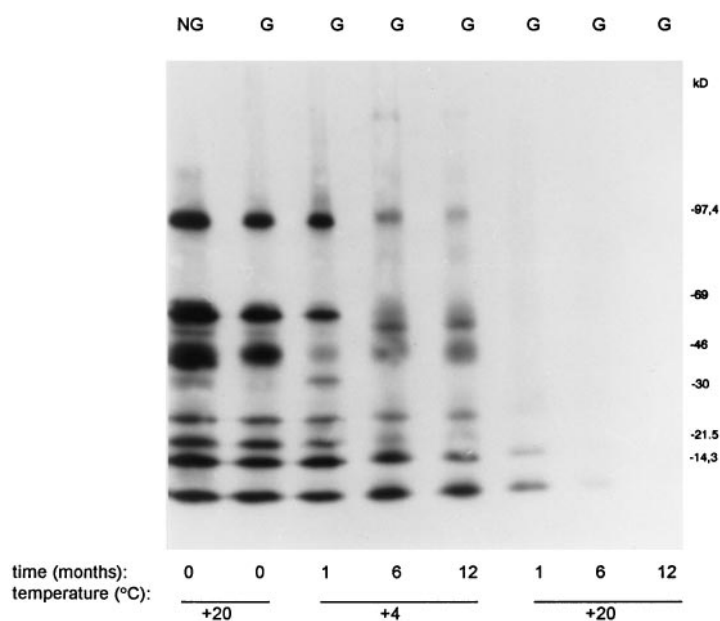


Fig. 2. IgE immunoblot of *P. ovale* extracts. G is 50% glycerol containing extract and NG is nonglycerol extract. Nonglycerolated extract, which was fresh, was not sterile filtered as all other extracts.

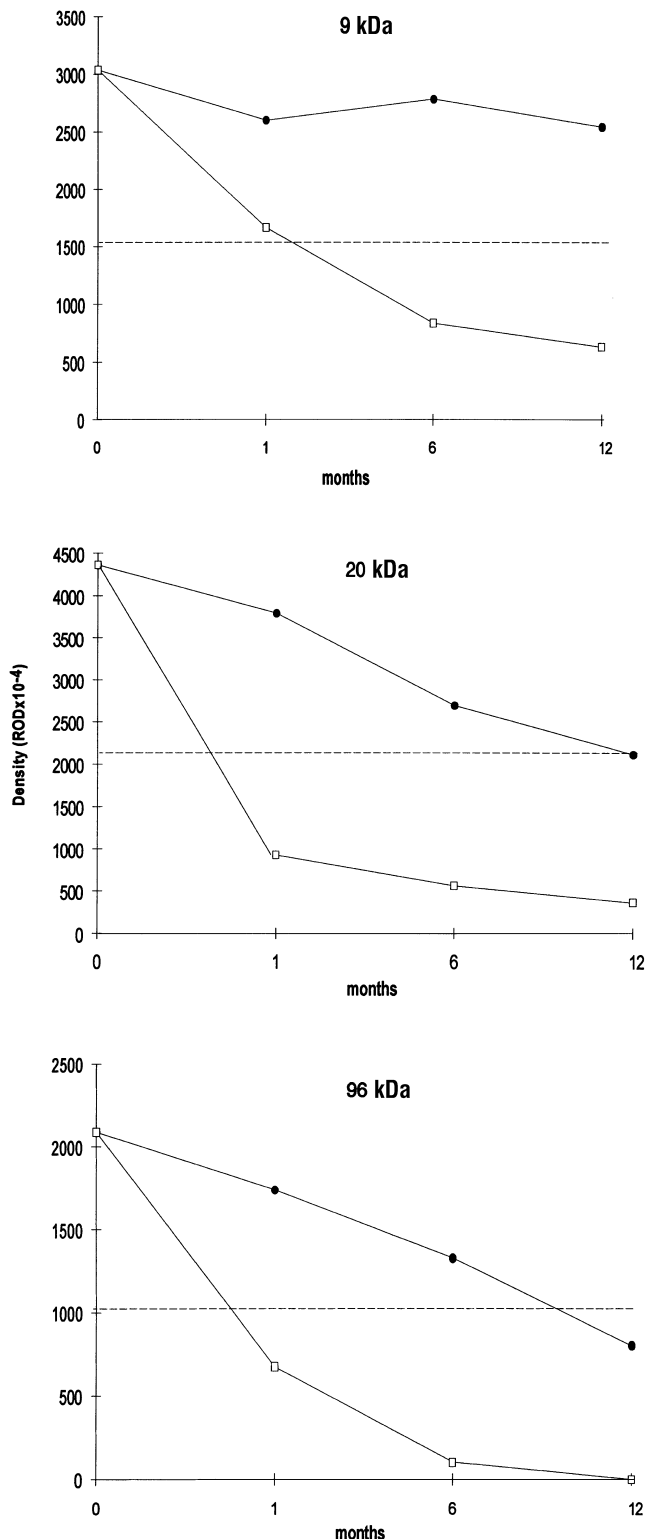


Fig. 3. The effect of temperature (● +4 °C and +20 °C) for the stability of important protein allergens: (a) 9 kDa, (b) 20 kDa and (c) 96 kDa. On the vertical axis density of the IgE immunoblots (ROD $\times 10^{-4}$) and on the horizontal axis the time of storage in months. Broken lines represent 50% degradation timepoints.

disappeared. After storage at +20 °C for one year in 50% glycerol there was a complete destruction of all the allergens (Fig. 2).

In the extract stored at +4 °C for 1 month in 50% glycerol, all important IgE-binding bands were relatively stable. After 6 months at +4 °C in 50% glycerol the 9, 20, 22, 27, 40, 50 and 96 kDa bands could be visualized. However, a new protein band of degradation productions in 180 kDa region had appeared. In extract stored for one year at +4 °C in 50% glycerol many important bands were seen, but most had been disappeared. The 9 and 20 kDa bands could be detected, but the 27, 40, 50 and 96 kDa bands had markedly weakened.

The loss in IgE binding activity was also seen in densitometry, especially when extracts were stored at +20 °C (Fig. 3). The stability of low molecular weight allergens was in general better than the stability of high molecular weight allergens. The 9 kDa band was the most stable main protein allergen retaining over 80% of its allergenicity when stored at +4 °C up to one year. At +20 °C the 9 kDa allergen lost 79% of its allergenicity in one year and the interpolated time point for 50% degradation was at 5–6 weeks. The 20 kDa allergen was clearly more labile than the 9 kDa allergen. After one year, the 20 kDa allergen lost 52% of its allergenicity when stored at +4 °C and 92% when stored at 20 °C. The 50% degradation time points were 2–3 weeks at +20 °C and 11–12 months at +4 °C. The very labile 96 kDa allergen lost 62% of its allergenicity during 1 year at +4 °C and 100% at +20 °C. The 50% degradation time points for the 96 kDa allergen were 2–3 weeks at +20 °C and 9–10 months at +4 °C.

Discussion

P. ovale is a lipophilic yeast, which grows as a saprophyte and an opportunistic pathogen on skin flora [18–20]. Generally the IgE response to *P. ovale* has been established with SPT, although no knowledge exists of the stability of *P. ovale* skin test allergens. According to our earlier studies the *P. ovale* allergen used in the SPT should include at least the 9, 20 and 96 kDa bands and mannan [12]. Therefore the serum pool used in this study to detect the IgE staining was chosen to contain samples with IgE antibody response especially directed against those bands. The selection was based on earlier immunoblotting and mannan-RAST studies with individual serum.

According to present results the allergens of *P. ovale* were very labile, especially when left at room temperature or warmer conditions. Majority of IgE staining disappeared already after storage for 1 month at room temperature. At +4 °C the stability was better and the samples contained some of the important allergens even after storage for 1 year. However, under normal SPT conditions the allergen

solutions are often kept at room temperature daily for several hours when the skin tests are performed. Under such circumstances the solutions may lose their IgE binding activity more rapidly.

Addition of 50% glycerol did not essentially improve the stability of the allergens in extracts stored at +4°C during one year. This clearly indicates that temperature of storage is more important than the stabilizer used.

During storage, in addition to disappearance of allergens, also new protein bands did appear. This was found for example in extracts which contained glycerol and were stored at +4°C for 6 or 12 months (Fig. 1). A new band was located in 180 kDa region and most probably represented degradation products from high molecular weight mannoprotein allergens, against which IgE antibodies were frequently found in our previous study [12].

When compared with the other yeasts, *P. ovale* allergens seemed to be as labile as *S. cerevisiae* allergens. For *S. cerevisiae* the storage for 1 month at +6°C in 50% glycerol extract destroyed two of the most important allergens, represented by 48 and 32 kDa bands [8]. The main 46 kDa allergen of *C. albicans* markedly lost its allergenicity in glycerol-free extract at +4°C already after 4 weeks. However, addition of glycerol improved the stability to 10 weeks. Three other allergens (29, 50 and 57 kDa) were stable after storage for 60 weeks [7]. Thus, *C. albicans* allergens seem to be more stable when compared to *P. ovale* allergens, but in general, all yeast allergen extracts are quite labile. These findings are not completely unexpected because of the high enzymatic activity in fungi.

It has been proposed earlier that the skin-prick test could be superior to other available assays for diagnosing type I reactions to *P. ovale* in AD patients [21]. According to the present results the stability of *P. ovale* skin test allergens was poor if the extract was stored at room temperature. That will decrease the value of the skin test especially if the SPT result is negative and the extract used is older than 1 month. In that case, freshly made allergen extract should be used or other tests, e.g. measurement of specific IgE antibodies in the serum of the patient, should be done.

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