

Composition and stability of allergenic extracts made from gamma-irradiated rye grass (*Lolium perenne*) pollen

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Summary

Background Phenol is commonly added to allergenic extracts as a bacteriostatic agent, but it is poisonous and also detrimental to proteins, which accelerates extract degradation. Sterilization by gamma-irradiation of the source material could be an alternative to the use of phenol.

Objective To analyse the potential effects of gamma-irradiation of pollen on the composition, potency, and stability of the resulting extract, and compare them with those of phenol.

Methods Ryegrass (*Lolium perenne*) pollen was sterilized by gamma-irradiation at a dose of 25 kGy. Extracts prepared from the irradiated pollen were then compared by electrophoresis techniques and RAST inhibition to extracts, without or with 0.5% phenol, from nonirradiated pollen. In addition, proteolytic activity was compared in extracts from irradiated and nonirradiated pollen. To evaluate the stability of extracts on storage, they were analysed after forced degradation for up to 7 days at 37°C.

Results When fresh extracts were analysed, there were no noticeable differences between the three types, as judged by immunoblotting and RAST inhibition experiments. However, on storage, extracts from irradiated pollen appeared to be superior to extracts from nonirradiated pollen, as some proteins were more stable in the former. This could be related to the lower proteolytic activity we have also observed in extracts from irradiated pollen. In contrast, extracts containing phenol degraded much faster, as proven by all our methods of investigation.

Conclusion Gamma-irradiation of pollen did not influence the IgE-binding capacity of the resulting extracts, but did yield extracts with somewhat improved stability, probably by reducing the proteolytic activity. It may be concluded that gamma-irradiation of the source material represents a good alternative to the use of phenol for the preparation of allergenic extracts.

Keywords: allergenic extract, electrophoresis, gamma-irradiation, phenol, protease, ryegrass pollen, stability

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Introduction

To prevent bacterial growth, phenol is added to allergenic extracts, sometimes already during the extraction step. However, a detrimental effect of phenol on allergens, that may play a role in the degradation and loss of activity of extracts during storage, has been described by several

groups [1–5]. We have demonstrated earlier that the addition of sucrose to the allergenic extracts can partly counteract this degrading effect [3].

Actually, phenol is added to extracts of not fully decontaminated source material. Another approach could be to first decontaminate the source material by gamma-irradiation and then prepare and store the resulting extracts in sterile conditions. This also would avoid bacterial growth, but without phenol and its degrading effects.

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Gamma-irradiation at high doses (25 kGy) is now routinely used in the medical industry to sterilize instruments. It is also used at relatively lower doses (generally up to 10 kGy) in the food industry, to reduce the microbial content of some products, or to delay or inhibit germination of seeds [6]. In both cases, the effects are the direct results of the destructive action of gamma-irradiation on DNA, which is the major target of ionizing radiation [7]. Conversely, proteins are less susceptible to direct effects, but rather are affected by the side products of irradiation. For example, water radiolysis liberates oxygen radicals that can oxidize proteins in solution [8,9]. Ozone, which is also produced consequently to irradiation, can react with proteins as well [10–12].

Because of such potential effects on proteins, it is important to study the consequences of gamma-irradiation of pollen on the quality of the resulting allergenic extracts, which has not been studied so far. This is of crucial interest in the perspective of using gamma-irradiation in the manufacture of allergenic extracts.

Therefore, the purpose of this work was to analyse extracts from gamma-irradiated ryegrass (*Lolium perenne*) pollen, and to compare them before and after storage with phenolated and non-phenolated extracts from nonirradiated ryegrass pollen.

Material and methods

Pollen

L. perenne pollen (batch number 021493901) was purchased from Allergon (Ängelholm, Sweden). It was 98% pure, contained less than 0.5% foreign pollen, and less than 0.4% mold spores, according to the manufacturer's data.

Irradiation

Three independent irradiations were carried out in a laboratory Cobalt-60 gamma-irradiator (Gammacell-220, Nordion, Ottawa, Canada). The pollen received a dose of 25 kGy, which is the minimal dose assuring sterility [6].

Extracts and forced degradation

Pollen extracts were prepared 1/10 (w/v) in water, or in 0.5% phenol, for 3 h at room temperature with continuous shaking. The pH of the mixture was constantly adjusted to 7.5 as described elsewhere [13]. After extraction, the extracts were centrifuged (17 500 g; 30 min, 4°C) before they were filtered through a 0.45- μ m membrane. Each extract was then divided into two parts. One part was stored in sterile vials at 37°C for up to 7 days (stored extract). The other part was immediately aliquoted and

kept at –20°C until use (fresh extract). After storage, extracts were filtered through a 0.45- μ m membrane to remove any precipitate, then aliquoted and frozen until use.

Patient serum pool

Fifty sera from grass pollen-sensitive patients were pooled to be used for immunoblotting and RAST-inhibition experiments. All sera had specific IgE to *L. perenne* pollen > 17.5 U_a/mL (CAP system, Pharmacia, Uppsala, Sweden), and total serum IgE < 2000 IU/mL (CAP system, Pharmacia). For the serum pool, specific IgE to *L. perenne* pollen was > 100 U_a/mL.

Protein content

The protein content of the extracts was determined by the Coomassie blue assay (Bio-Rad, Richmond, CA, USA), according to the manufacturer's instructions, with bovine serum albumin as a standard.

Electrophoresis

Isoelectric focusing (IEF), using an LKB 2117 Multiphor II (Pharmacia-biotech, Uppsala, Sweden), was carried out in 5% polyacrylamide thin gels containing 7.5% carrier ampholytes of pH range 3–10 (Isolab, Akron, OH, USA), poured on Gelbond PAG film (FMC, Rockland, ME, USA). The running conditions were 1500 V, 25 mA, and 15 W for a total of 1250 Vh.

SDS-PAGE was carried out in 12% polyacrylamide gels with a 5% polyacrylamide stacking gel, using the discontinuous buffer system of Laemmli [14]. Before application into the gel, samples were heated for 5 min at 100°C in sample buffer composed of 125 mmol/L TRIS, pH 6.8, 17.5% glycerol, 4% SDS, 1% β -mercaptoethanol, and 0.002% bromophenol blue. A voltage of 350 V was applied until the bromophenol blue reached the opposite side of the gel.

IEF and SDS-PAGE gels were either stained with Coomassie brilliant blue, or used for immunoblotting.

Immunoblotting

Proteins were transferred from IEF gels to PVDF membranes (Immobilon, Millipore, Bedford, MA, USA) by press blotting. Proteins in SDS-PAGE gels were electrophoretically transferred to PVDF membranes in transfer buffer composed of 25 mmol/L TRIS, 192 mmol/L glycine, and 20% methanol, at 100 V for 3 h. In both cases, the membranes were then immersed for 1 h in blocking solution, consisting of phosphate-buffered saline with 0.2% non-fat dried milk (PBS-NFDM 0.2%), before incubation overnight

at 4°C in serum diluted with PBS-NFDM 0.1% (125 µL serum/sample track on the membrane). Bound IgE was detected by monoclonal mouse antihuman IgE antibodies (CLB, Amsterdam, The Netherlands; 1 µL antibody solution/sample), 1 h at room temperature, followed by peroxidase-conjugated goat antimouse IgG antibodies (CLB; 0.5 µL antibody solution/sample), 2 h at room temperature. All dilutions and washes were made with PBS-NFDM 0.1%. Bands were revealed by incubation in the peroxidase substrate TMB (KPL, Gaithersburg, MD, USA). The parts of the membranes containing the markers were extensively rinsed in distilled water immediately after blotting, dried out, and then stained with sulforhodamine [15].

RAST inhibition

Several dilutions of allergenic extract (50 µL) were each incubated at room temperature with 50 µL of serum pool in a microtitre plate. The starting dilutions were 1 : 2000 for the fresh extracts, 1 : 1300 for the extracts stored for 1 day, 1 : 900 for the extracts stored for 3 days, and 1 : 200 for the extracts stored for 7 days at 37°C, each followed by five consecutive dilutions (extract : buffer — 3 : 2). All dilutions were made in PBS containing 1% human serum albumin. After 2 h, *L. perenne*-coupled CAPs (Pharmacia) were added to the wells and incubated for 30 min. The CAPs then were washed and incubated for 2 h 30 min with 50 µL radioiodinated anti-IgE antibodies (Pharmacia). They were washed before reading in a gamma-counter. Every dilution of every sample was tested in duplicate. Relative potencies were calculated according to Anderson and Baer [16]. The fresh non-phenolated extract from nonirradiated pollen was used as reference.

Proteolytic activity

The proteolytic activity of fresh extracts prepared from irradiated pollen and of fresh extracts from nonirradiated pollen was determined by an assay using resorufin-labelled casein as a universal protease substrate (Ref. 1080733, Boehringer-Mannheim, Mannheim, Germany). Briefly, 50 µL of 0.4% resorufin-labelled casein (w/v), 100 µL of allergenic extract, and 50 µL of 0.5 mol/L TRIS-HCl pH 7.8, 0.02 mol/L calcium chloride were all mixed and incubated for 6 h at 37°C.

Resorufin-labelled peptides were released from resorufin-labelled casein by the action of the proteases present in the allergenic extract. The reaction was stopped by the addition of 480 µL of 5% trichloroacetic acid before the mixture was centrifuged at 12 000 g for 6 min. Resorufin-labelled casein was precipitated, while resorufin-labelled peptides remained in the supernatant. The concentration of those peptides, as

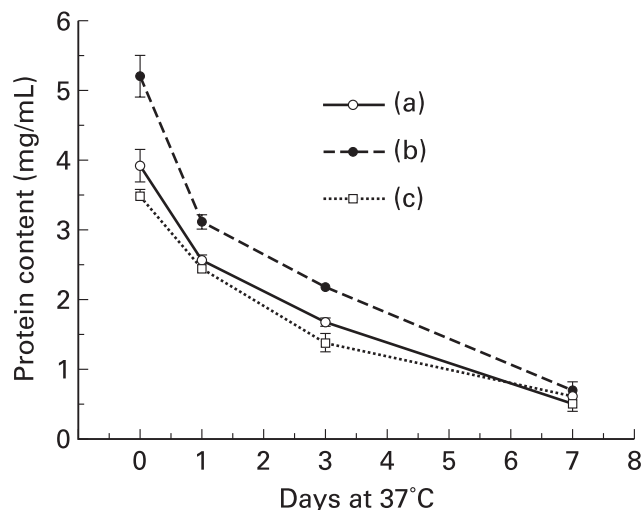


Fig. 1. Protein content by Coomassie brilliant blue method of extracts prepared from nonirradiated ryegrass pollen without (a) or with 0.5% phenol (c), and from irradiated ryegrass pollen without phenol (b), stored for up to 7 days at 37°C. Mean $\pm$ SEM of three experiments.

measured by absorbance at 574 nm, reflected the proteolytic activity present in the sample.

Results

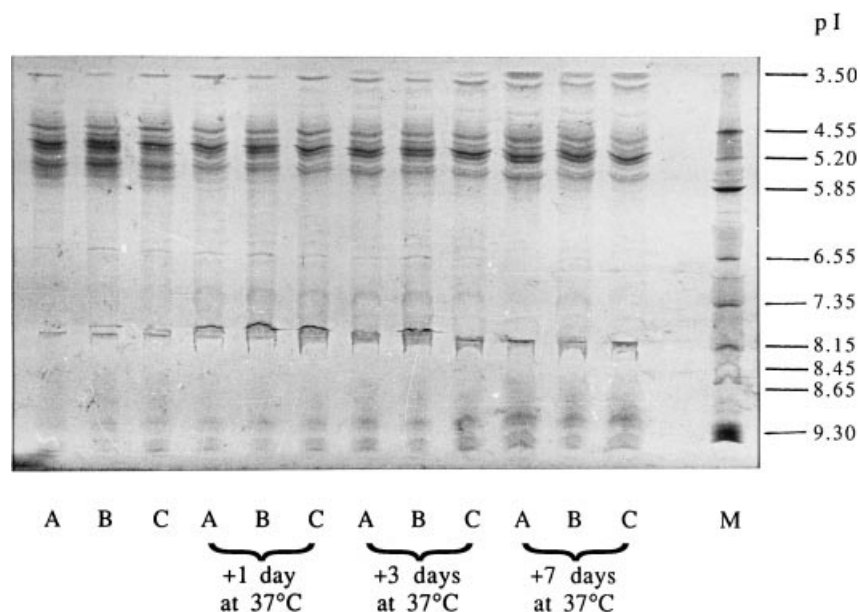
Total protein analyses

Protein content Protein content was measured in the fresh extracts, and in the extracts stored for 1, 3 and 7 days at 37°C. Figure 1 shows that irradiated pollen gave the highest extraction yield. Even though an extract from irradiated pollen seemed to degrade somewhat faster during the first day at 37°C, its protein content remained superior to that of extracts from nonirradiated pollen until at least 3 days of storage. The presence of 0.5% phenol did not influence much the protein content of extracts from nonirradiated pollen. After 7 days at 37°C, all extracts had approximately the same protein content.

Protein profile in IEF gels The three extracts showed no obvious qualitative difference in Coomassie blue-stained IEF gels, neither as fresh extracts, nor during storage at 37°C (Fig. 2).

The bands in the pI region 4.5–5.9 were somewhat fainter in all extracts after 1 day of forced degradation at 37°C. A band at pI-3.5 appeared in the three types of extract from the first day of storage, and was still well visible after 7 days at 37°C. A thin band at pI-6.7, initially present in all extracts, remained visible in the extract from irradiated pollen after 7 days at 37°C, but not in extracts from nonirradiated pollen.

Fig. 2. Coomassie brilliant blue-stained IEF gel. A: extract prepared from nonirradiated ryegrass pollen without phenol; B: extract prepared from irradiated ryegrass pollen without phenol; C: extract prepared from nonirradiated ryegrass pollen with 0.5% phenol. Extracts are shown before and after storage for 1, 3, and 7 days at 37°C. Fifteen micrograms of protein was applied per lane; pI markers are on lane M.



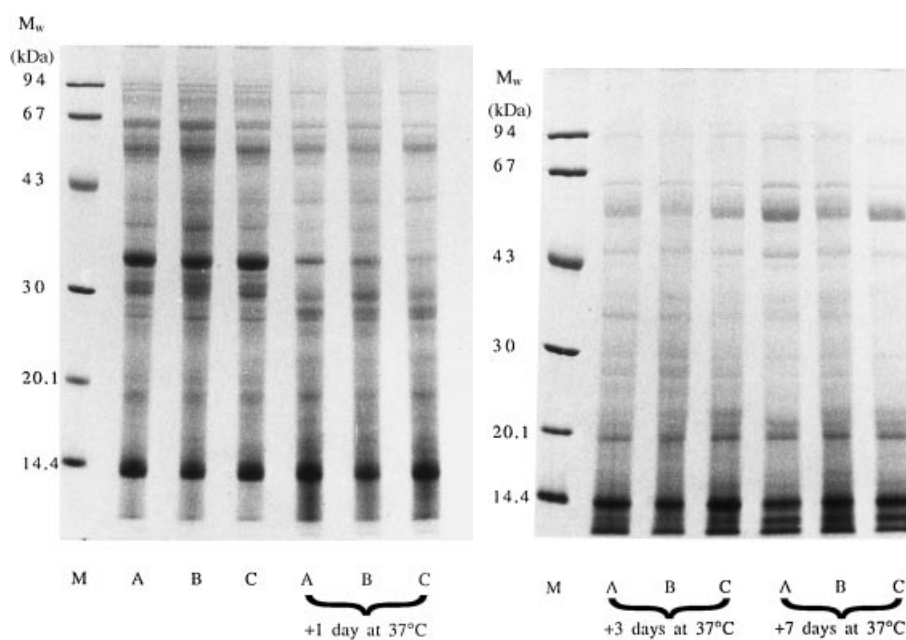
A set of protein bands in the basic region of the patterns, at pI 9–9.3, became more intense as the extracts were stored. This may relate to the accumulation of basic degradation fragments, but also to the visualization of stable proteins. Indeed, to make qualitative comparisons of extracts, the same total amount of protein must be applied in the gel for every sample. Since fresh extracts had higher protein concentrations, they had to be diluted to meet the lowest concentration of stored extracts. Therefore, the bands corresponding with stable proteins logically appeared with less intensity in the patterns of the fresh extracts than in those of the corresponding stored extracts.

Protein profile in SDS-PAGE gels In Coomassie blue-stained SDS-PAGE gels, the three types of extract displayed similar patterns of 23 bands, ranging from approximately 13 to 100 kDa (Fig. 3).

After 1 day at 37°C, some qualitative differences were observed, in comparison with the fresh extracts. Indeed, the majority of the bands faded, while some completely vanished (at 79, 38, 32, and 31 kDa). The band at 36 kDa, likely to correspond with Lol p 1, lost relatively more intensity than the rest of the pattern.

After storage for 3 days, the SDS-PAGE patterns of the extracts showed further fading, except maybe in the region

Fig. 3. Coomassie brilliant blue-stained SDS-PAGE gels. A: extract prepared from nonirradiated ryegrass pollen without phenol; B: extract prepared from irradiated ryegrass pollen without phenol; C: extract prepared from nonirradiated ryegrass pollen with 0.5% phenol. Extracts are shown before and after storage for 1, 3 and 7 days at 37°C. Twenty micrograms of protein was applied to each lane. Molecular mass markers are on lane M.



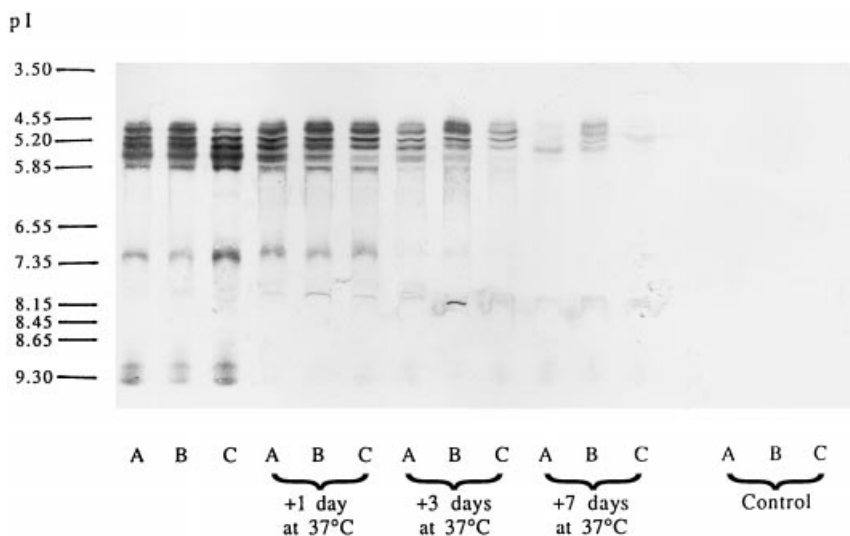


Fig. 4. IgE immunoblot from IEF gel. A: extract prepared from nonirradiated ryegrass pollen without phenol; B: extract prepared from irradiated ryegrass pollen without phenol; C: extract prepared from nonirradiated ryegrass pollen with 0.5% phenol. Extracts are shown before and after storage for 1, 3 and 7 days at 37°C. Extracts before storage were also incubated with buffer instead of serum (control). Seven micrograms of protein was applied to each lane. pI markers are indicated on left.

of the lowest molecular mass, but this is likely to be attributed to the increasing amount of intermediate degradation products. The band corresponding with Lol p 1 became very faint, and almost invisible for the phenolated extract.

After 7 days of storage, more bands disappeared from the profiles. The band corresponding with Lol p 1 totally faded out in the pattern of the phenolated extract. In contrast, two bands at 22.5 and 20.5 kDa were more prominent for the phenolated extract. The bands with a high molecular mass that were more intense in stored extracts than in fresh extracts were likely to relate to stable proteins, as already explained for Coomassie blue-stained IEF gels.

The intense low-molecular mass bands, of less than 14 kDa, might relate to degradation products.

IgE binding analyses

Allergen detection in IEF patterns An IgE immunoblot from an IEF gel is shown in Fig. 4. For all three fresh extracts, a strong IgE reactivity was demonstrated for six bands in the pI region 4.5–5.9 (possibly Lol p 1, Lol p 2 and Lol p 11), one band at pI-7 (possibly Lol p 5), and two bands in the basic pI region 9–9.3 (possibly Lol p 4). Additional bands at pI-6.2, 6.45 and 8.6 were visible in the pattern of the fresh phenolated extracts only.

For all three extracts the bands in the pI region 4.5–5.9 progressively faded as the extracts were stored. After 7 days at 37°C, however, IgE-binding in this pI region remained clearly more intense for the extract prepared from irradiated pollen than for the two other types of extract. The band at pI-7 progressively faded and finally disappeared in the three patterns of extracts stored for 7 days at 37°C. The two bands in the basic region almost disappeared after 1 day of storage at 37°C. Only a slight staining at this place still remained in

the immunoblot pattern of the three types of extract up to the seventh day of storage. The bands that were specific for the fresh phenolated extract disappeared in the extract stored for 1 day at 37°C.

Allergen detection in SDS-PAGE patterns An IgE immunoblot from an SDS-PAGE gel is shown in Fig. 5. Eleven bands reacting with IgE were detected in the patterns of all three types of fresh extracts. However, the band at 66 kDa was almost invisible in the phenolated extract. Control strips, incubated in buffer instead of serum, showed no band (not included in the figure).

The patterns of the extracts stored for 1 day demonstrated dramatic changes in the high molecular mass region. Particularly, the very reactive band at 42 kDa, but also the thin bands between 36 and 42 kDa, as well as a band at 33 kDa, completely disappeared from these extracts. The higher molecular mass bands, including the one possibly corresponding to Lol p 4 at 57 kDa, were only slightly visible in the two non-phenolated extracts, and not visible at all in the phenolated extract. The increased intensity, as compared with the fresh extracts, of the two bands at 26 and 28 kDa (possibly Lol p 5) may be due to the dilution effect, as already described in the former paragraph.

In the patterns of the extracts stored for 3 days at 37°C, only four bands were still detected by IgE. The protein at 28 kDa is clearly better preserved in the extract from irradiated pollen. For this latter type of extract only, a barely visible staining was still present in the high molecular mass range of the pattern.

After storage for 7 days, the extract from irradiated pollen displayed the highest IgE reactivity to the remaining detectable bands, whereas all bands were fainter in the pattern of the phenolated extract.

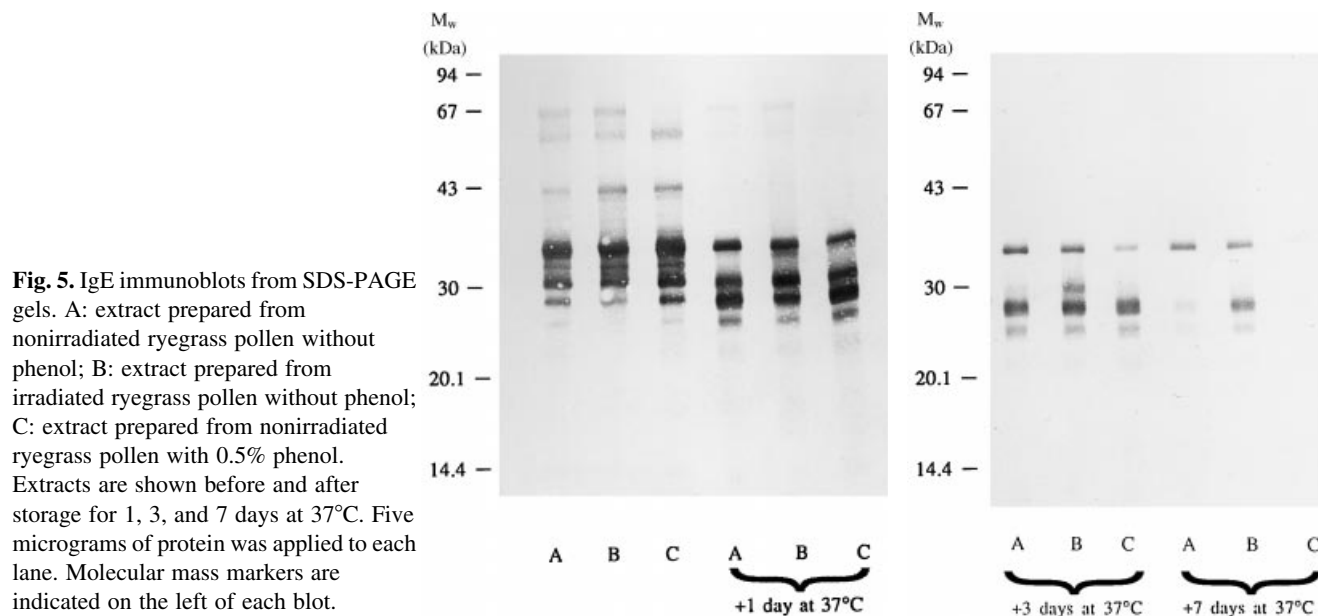


Fig. 5. IgE immunoblots from SDS-PAGE gels. A: extract prepared from nonirradiated ryegrass pollen without phenol; B: extract prepared from irradiated ryegrass pollen without phenol; C: extract prepared from nonirradiated ryegrass pollen with 0.5% phenol. Extracts are shown before and after storage for 1, 3, and 7 days at 37°C. Five micrograms of protein was applied to each lane. Molecular mass markers are indicated on the left of each blot.

RAST-inhibition The results are shown in Table 1. Immediately after extraction, the non-phenolated extracts were almost equally potent, whereas the phenolated extract tended to be somewhat less active. On storage, both types of non-phenolated extracts showed a parallel decrease in activity. The loss of potency of the phenolated extract seemed to be more dramatic. Indeed, only 8.3% of its original potency still remained after one day of storage, in comparison with 17.2 and 18.6% for the non-phenolated extracts from nonirradiated and irradiated pollen, respectively. After 7 days of storage, all three types of extracts performed almost equally.

Proteolytic activity

The results are shown in Fig. 6. Protease activity in fresh

extracts of irradiated pollen was decreased by 19, 22.9 and 17% after three independent irradiations, respectively, compared with fresh extracts of nonirradiated pollen.

Discussion

To determine whether gamma-irradiation of pollen influences the composition and stability of the resulting allergenic extracts, we have compared extracts prepared from irradiated and nonirradiated pollen. Because gamma-irradiation could replace the use of phenol, phenolated extracts from nonirradiated pollen were also included in the comparison.

SDS-PAGE and IEF Coomassie blue-stained gels and corresponding immunoblots did not show clear qualitative differences between the three types of fresh extracts.

Table 1. Results of RAST-inhibition studies

	Relative potency		
	Extract A	Extract B	Extract C
Fresh	1	0.927 ± 0.211	0.746 ± 0.234
+ 1 day of storage	0.172 ± 0.007 (17.2%)	0.169 ± 0.03 (18.6%)	0.062 ± 0.023 (8.3%)
+ 3 days of storage	0.053 ± 0.01 (5.3%)	0.081 ± 0.011 (8.9%)	0.022 ± 0.005 (2.9%)
+ 7 days of storage	0.045 ± 0.01 (4.5%)	0.042 ± 0.009 (4.6%)	0.031 ± 0.001 (4.2%)*

Extract A: prepared from nonirradiated ryegrass pollen; extract B: prepared from gamma-irradiated ryegrass pollen; extract C: prepared from nonirradiated ryegrass pollen with 0.5% phenol. The fresh non-phenolated extract of nonirradiated pollen (extract A) was used as reference. The values are the means ± SEM of three independent experiments with different extracts (*mean of two experiments). The numbers in parentheses represent the remaining potency compared with the corresponding fresh extract. Friedman's two-way analysis of variance: $P < 0.05$.

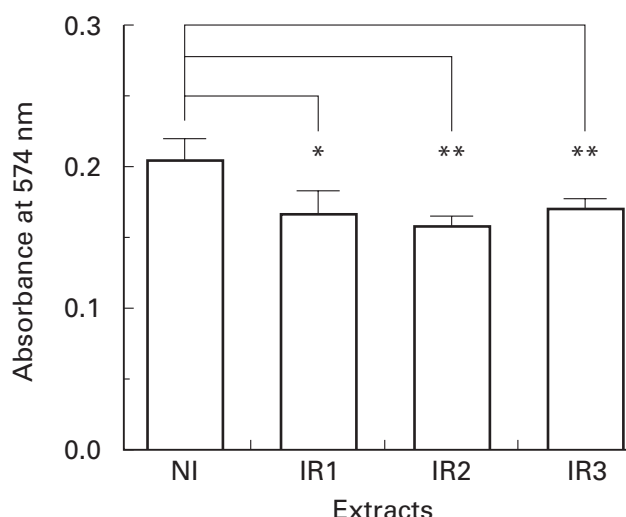


Fig. 6. Proteolytic activity of allergenic extracts reflected by absorbance at 574 nm (see Material and methods). NI: extract from non irradiated pollen. IR1, IR2, IR3: extracts from irradiations 1, 2 and 3. Values are means $\pm$ SD of five independent extracts. * P < 0.05. ** P < 0.01 (Mann–Whitney U -test).

However, the extraction yield was higher with the gamma-irradiated pollen. This could be related to the fact that irradiation has reduced the proteolytic activity in the extracts. Pollen extracts are known not to contain a lot of proteases [17], but trypsin activity has been demonstrated in *Lolium perenne* extracts [18], which is likely to be active during extraction. In our study the protease activity in extracts from irradiated pollen was 17–22.9% lower than in extracts from nonirradiated pollen. Reduction of enzymatic activity by irradiation has already been described [19,20]. When irradiated, enzymes in the dry state, as is the case in freeze-dried pollen, may undergo structural changes resulting from tryptophan destruction [21] or breakage of disulphide bridges [19,20], which might explain their loss of activity.

If fresh extracts seemed to be similar, differences in allergen composition and in potency became evident on storage at 37°C. Particularly, the phenolated extract demonstrated the most dramatic loss of quality, as shown by IEF and SDS-PAGE immunoblot analyses. The degradation of the phenolated extract looked even more pronounced when analysed by RAST-inhibition. Such observation is likely to refer to the detrimental effect of phenol on proteins, already described for various extracts [1–5]. However, the effect of phenol in the present study seemed less pronounced than in our previous study [3]. This might be explained by differences in the pH of the solutions. In the present work, the pH has been continuously monitored and adjusted to 7.5, if necessary, during extraction,

which was not the case in our previous study. The more dramatic degradation we previously observed was thus most likely the result of a combined effect of pH variation and phenol. The present study shows the influence of phenol only.

At this point, we must report that the results in immunoblots did not always match those obtained by Coomassie blue staining, for which the differences between the extracts were less noticeable. This is probably because a given band in a pattern may be composed of various proteins, among them allergens. Even if those allergens are destroyed, as proven by immunoblots, there might still be enough other nonallergenic protein in the same band to be detected by Coomassie blue. Another phenomenon, particularly relevant in the case of our IEF gels that are made in non-denaturing conditions, is that proteins still can be found at the same position in the gel, since their global charge is unchanged, even though their 3D-structure can be somewhat affected. Therefore, it may happen that allergens still are detected by Coomassie blue in gel, but not by antibodies in blots, simply because essential conformational epitopes have been destroyed. This does not apply to SDS-PAGE gels in reducing conditions, where only the so-called linear epitopes remain.

When comparing the two types of non-phenolated extracts on storage, it appeared that those made from gamma-irradiated pollen were slightly superior to those from nonirradiated pollen. Particularly, some proteins seemed to be better preserved in the former, as shown by immunoblots from IEF and SDS-PAGE gels. Here again, this can logically be related to the decrease in protease activity in the extracts from irradiated pollen.

It will be interesting to study these effects of irradiation on other allergenic raw material, especially those containing greater amounts of proteases, such as house dust mite and moulds. If such effects could be confirmed in any source material, this would be of particular importance in allergen mixtures, where proteases have been shown to play a major role in the degradation of allergens [17].

We have demonstrated in this study that gamma-irradiation of ryegrass pollen, at a sterilizing dose of 25 kGy, did not impair the composition and IgE-binding capacity of the resulting extracts, although irradiated antigens are likely to show some structural changes. Such changes, however, seemed to be sufficient to affect enzymatic activity, which consequently gave a beneficial effect on the stability of the allergenic extracts. In contrast, to reach the same goal, that is prevention of bacterial growth, the use of phenol was damaging to the extracts. Although phenol will remain necessary in multipuncture vials, it may be envisaged to avoid its use, at least in the first steps of preparation of an extract or in single-use vials, by irradiating the source material.

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