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Influence of Cultivar and Processing on Cherry (*Prunus avium*) Allergenicity

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Oral allergy syndrome is an immediate food allergic event that affects lips, mouth, and pharynx, is often triggered by fruits and vegetables, and may be associated with pollinosis. Here, we report on the allergenic pattern of different varieties of cherry (*Prunus avium*) and results obtained by applying several technological processes to the selected varieties. Whole cherries were submitted to chemical peeling, thermal treatment, and syruping processes, and the relative protein extracts were analyzed by in vitro (sodium dodecyl sulfate−polyacrylamide gel electrophoresis and immunoblotting analysis) and in vivo tests (skin prick test). Electrophoretic analyses demonstrated that there was no marked difference among cherry cultivars. Chemical peeling successfully removed Pru av 3, a lipid transfer protein (LTP) responsible for oral allergy syndrome in patients without pollinosis, leading to the industrial production of cherry hypoallergenic derivatives. Furthermore, the syruping process removed almost all allergenic proteins to whom patients with pollinosis are responsive. In vivo tests confirmed electrophoretic results.

KEYWORDS: ***Prunus avium*; allergy; fruit processing; lipid transfer protein; syruping**

INTRODUCTION

Food allergy represents a big challenge in Western society for the ever-increasing number of people involved as well as for the severity of the reactions, given that in the past decade it has caused the major number of fatal or near-fatal anaphylactic reactions (*1*). The importance and widespread interest that this disease and the elimination diet have obtained has forced the onset of Directive 2003/89/EC (*2*) that deals with the indication on the label of all the allergenic ingredients present in foodstuff. This directive is very important also because complete avoidance of the offending food is at present the only way to prevent food allergic reactions. Important goals for scientific research in food allergy are, however, the characterization of the largest possible number of food allergens and the study of their stability to technological processes, in order to point out potential modifications that can remove the IgE-binding properties from native food. This could allow the preparation of food products that lack allergenic potential, as previously demonstrated for peach allergy (*3*). Given that different allergenic proteins with different physical−chemical properties are present in each food, it is conceivable that only a patient-tailored procedure can produce useful results. For example, when people allergic to fruits and vegetables are considered, we can distinguish three different scenarios on the basis of the class of the causative proteins. The patients can be affected by birch fruit syndrome, due to Bet v 1 homologous allergens, or by the lipid transfer protein (LTP) syndrome, due to sensitization to homologous LTP in vegetables foods, or by the latex fruit syndrome, due to the sensitization to hevein-like domains in food (*4*).

It is thus necessary to identify the kind of sensitizing proteins and their specific suitability to technological modifications in order to find the proper solution for different patients and prepare products that respond to different requests. For example, we know that in hypersensitivity to Bet v 1 homologues the allergenicity of fruits and vegetables can be reduced by high-temperature treatment, as has been demonstrated for Cor a 1.04 in hazelnut (*5*, *6*). On the contrary, LTP allergens are not sensitive to high temperature (*3*, *4*) and only the elimination of the peel can allow the intolerant patient to eat the culprit foods. Different procedures can thus produce different hypoallergenic products, improving the nutrition opportunities for allergic subjects.

The present study should be considered in this perspective, that is, the possibility of obtaining hypoallergenic cherry derivatives for cherry-allergic patients who suffer from LTP syndrome. Four different allergens have been recently identified in cherry: Pru av 1 (*7*) and Pru av 4 (*8*) are proteins homologous

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Table 1. Clinical Information on the Patients in the Study[a]

patient	age	sex	spt cherry	rast cherry	pollinosis	other foods causing symptoms
1	36	M	++++	6.08	grass	Prunoideae, apple, tomato
2	28	M	+++	4.12	//	Prunoideae
3	32	F	+++	2.53	grass, ragweed	Prunoideae, apple, walnut, peanut
4	27	M	+++	1.85	//	Prunoideae, apple
5.	41	F	++++	34.7	//	Prunoideae
6	32	F	+++	3.31	grass	Prunoideae
7	21	M	++++	8.41	//	Prunoideae, apple, peanut
8	38	F	+++	1.82	birch	apple
9	35	F	+++	8.04	birch, grass	peach, apple, melon
10	27	F	+++	5.85	birch, grass	peach, apple
11	34	F	+++	3.25	birch	peach, apple
12	25	F	+++	1.84	birch, grass	peach, apple

[a] The first seven patients were exclusively LTP-positive. The other five patients (8–12) were suffering from birch pollinosis and their sera were used for the birch-positive pool, two of them (9, 12) were also LTP-positive.

to major birch allergens, Bet v 1 and Bet v 2 respectively. These proteins are responsible for birch and cherry cross-reactivity in the birch fruit syndrome that is frequent, particularly in central and northern Europe. Other major allergens are Pru av 3 (*9*), an LTP that is highly homologous to peach LTP, and Pru av 2 (*10*, *11*), which belongs to thaumatin-like proteins of family 5 of pathogenesis-related (PR) proteins. Pru av 3 is an important allergen for people living in the Mediterranean area and its strong allergenicity was recently related to its high stability against thermal processes and digestion (*9*). The aim of our study was to detect the allergenic pattern of six different cherry cultivars and identify technological procedures able to decrease in particular the strong allergenicity of cherry Pru av 3 in different cherry cultivars in patients affected by the so-called LTP Syndrome, which is a particular kind of oral allergy syndrome due to the consumption of LTP-containing foods.

MATERIALS AND METHODS

Samples. Six cultivars of sweet cherry (*Prunus avium*) were harvested when fully ripe in an orchard near Vignola (Modena, Italy) and treated within 24 h of receiving. Black peel and flesh variety (Mora di Vignola and Durone Nero I di Vignola), yellow peel and flesh variety (Napoleon and Rainier), and pink peel and flesh variety (Adriana and Grace Star), were considered. Whole (TQ) or chemically peeled (P) cherries were submitted to different processing in order to obtain several technological derivatives. One commercial sample of sweet cherries in syrup was also analyzed.

Patients. All the in vitro tests were performed with sera of individuals selected on the basis of a history of oral allergy syndrome (OAS), positive skin prick tests, and circulating IgE-specific antibodies for fresh cherry (CAP-RAST, Pharmacia Diagnostics AB, Sweden). Clinical information on the selected patients is presented in **Table 1**. For our purposes, patients were divided in two groups; in the first one patients were exclusively LTP-positive (1−7), and in the second pool patients were suffering from birch pollinosis (8−12), and two of them (9 and 12) were also LTP-positive. The separation was made on the basis of SDS−PAGE immunoblotting results and of our previous studies in which patients allergic to Prunoideae fruits without birch pollinosis were exclusively positive to LTP (*3*).

Chemical Peeling. The procedure described by Brenna et al. (*3*) was followed, with minor changes. Briefly, cherries were dipped in 10% (w/v) NaOH at 70 °C for about 30−60 s, according to fruit firmness, then rinsed and gently rubbed under tap water. The peeled cherries were then briefly immersed in 1% (w/v) citric acid to restore their physiological acidic pH.

Thermal Treatment. Whole and peeled fruits were pitted and homogenized in a blender and then poured into 460 mL tinplate cans, which were sealed with a lab-scale seamer, model A1 (Bertuzzi SpA, Brugherio, Italy), and autoclaved at 100 °C for 5 min.

Syruping Process. Whole and peeled cherries were immersed for 2 weeks in 1 M citric acid (pH 2.5), containing $CaCl_2$ 1 g/L and $Na_2S_2O_5$ 10 g/L (fruit/solution 1.5). After this step a portion of whole and peeled cherries was subjected to protein extraction as described below. Remaining cherries were submitted to a standard syruping procedure that requires the removal of naturally present anthocyanins in order to obtain a final product with a color stable over time. So cherries were dipped in 0.5% (w/v) citric acid, heated to 90−95 °C, and kept at this temperature for 3 min. Cherries were transferred into a cold solution containing 0.3 g/L erythrosine (E 127), then heated to 90−95 °C for 3 min and left in the dyeing solution for about 1 h. Following cycles of heating in acidic conditions to fix color and remove the dye excess, cherries were finally sealed in jars with a saccharose syrup (40%) and heated to 85−87 °C for 10−15 min (*12*).

Protein Extracts. Whole and peeled fruits, both fresh and thermally treated, experimental and commercial cherries in syrup were treated according to Brenna et al. (*13*), with minor changes. Samples were pitted (if not yet done), homogenized (2:1 w/w) in 10 mM sodium phosphate buffer containing 0.5 M NaCl, 0.2% (w/v) $Na_2S_2O_5$, and 2% (w/v) cross-linked poly(vinyl polypyrrolidone) (PVPP; Divergan EF, BASF, Ludwighafen, Germany). After centrifugation at 6000 rpm for 20 min, the supernatant was dialyzed (membrane cutoff 6−8 kDa; Spectra/Por 1, Spectrum, Laguna Hills, CA) against distilled water containing 40 mg/L NaF. The dialysate was added at 4 mL/100 mL of suspension of 50% PVPP in distilled water and, after stirring for 15 min, filtered and lyophilized. Before analyses, protein extracts were dissolved in a volume of distilled water corresponding to $^1/_{10}$ of the initial sample volume. Protein determination was performed in accord with Bradford (*14*).

SDS−PAGE and Immunoblotting. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out essentially as described by Neville (*15*) with a polyacrylamide stacking gel (6%) on top of a gradient separation gel (7.5−20%). Samples were previously diluted 1:2 with a denaturating buffer, containing 3% 2-mercaptoethanol, 2% SDS, 7% glycerol, and 0.001% bromophenol blue as tracking dye, and heated in a boiling water bath for 5 min. An aliquot (200 μL) of each extract, diluted as above, was run in any 2.5 cm well. The electrophoresis run was performed at 6 mA/gel for 18−20 h in a Protean II xi vertical electrophoresis cell (Bio-Rad Laboratories Inc., Hercules, CA). Proteins were stained with Coomassie Brilliant Blue R-250.

After separation, proteins were electrophoretically transferred onto a nitrocellulose sheet (Amersham, Buckinghamshire, U.K.) and incubated overnight with the sera under investigation, that is, patients with cherry allergy alone or with cherry allergy and birch pollinosis. The sheets were then washed and incubated overnight with ^{125}I-labeled anti-IgE (CAP RAST IgE RIA, Pharmacia & Upjohn, Uppsala, Sweden). Following final washings, the sheets were dried and left in contact with a photographic plate (Hyperfilm, Amersham) at −70 °C for 5 days.

Skin Prick Test with Different Experimental Cherry Extracts. Patients presenting only a cherry allergy without sensitization to birch pollen underwent skin prick tests with extracts from whole and chemically peeled cherries, representing all the typologies of cultivars considered in this study and, for the Napoleon variety, its derivatives in syrup (whole and peeled). Extracts were obtained by centrifuging homogenized fruit at 6000 rpm for 20 min and then diluting (1:1 v/v) the supernatant with glycerol (Alleanza Salute SpA, Rome, Italy). Skin testing was performed on the volar aspect of the forearm with a monodentate lancet; histamine (1%) and saline solutions were used as positive and negative control, respectively. Tests were considered positive if the weal produced had a mean diameter of at least 3 mm (*16*). Results were transferred to a paper sheet by placing transparent tape over the weal and flare and marking the size of the weal with a felt-tipped pen. After scanning, areas of weals were evaluated with Adobe Photoshop Elements 2.0 (Adobe Systems Inc., San Jose, CA). Student's *t*-test was performed with Statgraphics Plus 4.0 (Herdon, VA).

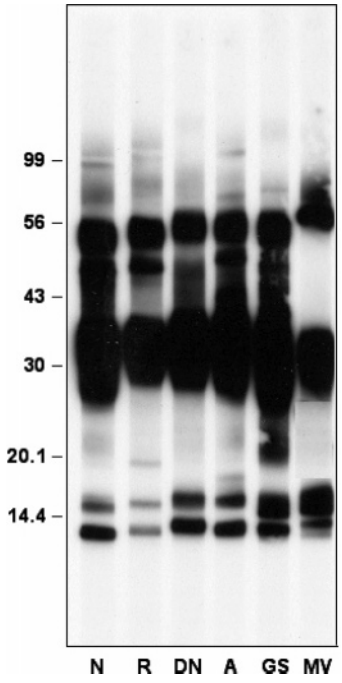

Figure 1. Immunoblotting of raw whole cherry cultivars analyzed. N, Napoleon; R, Rainier; DN, Durone Nero I di Vignola; A, Adriana; GS, Grace Star; MV, Mora di Vignola. Immunoblottings were performed with the birch-positive pool sera.

Purification of 9 kDa Protein from Cherry by HPLC. The LTP protein from raw concentrated cherry was purified (unpublished data) by analytical cation-exchange chromatography with an HPLC system (AKTA Purifier; Pharmacia Biotech, Uppsala, Sweden) on a Resource S (6 mL) column (Pharmacia Biotech). The column was eluted with a salt gradient from 0 to 1 M NaCl in 0.03 M sodium citrate buffer (pH 2.2). To obtain the pure 9 kDa component, a second purification step was performed, loading the concentrated sample onto a Hiloded Superdex 75 (Pharmacia Biotech) equilibrated and eluted with 0.15 M NaCl in 0.03 M sodium citrate buffer (pH 4.7−5). The chromatogram was monitored at 280 nm. The third peak, containing the 9 kDa protein, was analyzed by SDS−PAGE and immunoblotting with pooled sera of patients allergic to cherry. We verified this 9 kDa protein was cherry LTP by inhibiting it with an in-house lyophilized extract of cherry LTP.

Immunoblotting Inhibition Experiment. To demonstrate that the 9 kDa allergen component identified in raw Napoleon cherry (TQ) and other cultivars by immunoblotting corresponded to cherry LTP, an immunoblotting inhibition experiment was performed. Briefly, 0.5 mL samples containing 40 or 4 μg of purified cherry LTP were incubated with 0.5 mL of pooled sera and 1 mL of phosphate buffer. After 1 h the nitrocellulose strips of the raw cherry electroblot were incubated with preadsorbed sera overnight. Immunodetection was done as described above.

RESULTS AND DISCUSSION

Different degrees of allergenicity may depend, for the same vegetable species, on cultivar and agronomic practices, such as found for apples (*17*, *18*), bell peppers (*19*), and dates (*20*). In this study we considered several cultivars of cherry, characterized by different phenotypes, in order to point out, if possible, naturally hypoallergenic fruits.

SDS−PAGE of any of the four extracts (whole and peeled, with and without thermal treatment) for each of the six cultivars did not present any relevant difference in the protein bands, considering either their pattern or intensity (data not shown). We report in **Figure 1** the immunoblotting results of the six fresh whole cherry cultivars analyzed. It is evident that a large

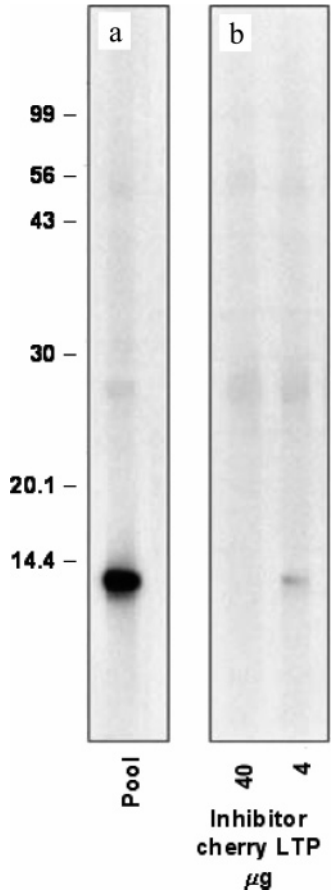

Figure 2. (**a**) Immunoblotting of whole (TQ) cherry Napoleon extract performed with pooled sera of patients positive for LTP only (1–7). (**b**) Immunoblotting inhibition performed with different amounts of purified cherry LTP. With 40 μg the binding is completely inhibited, while a low level of radiostaining is detectable at 4 μg.

number of allergenic proteins are present in the whole range of molecular masses considered. In general terms, we can affirm that the allergenic pattern is very similar among the cultivars chosen, with minor differences, which did not seem to be correlated with their phenotype. In particular, all of them presented clear bands, near 9, 14, 30, 45, and 67 kDa, besides other minor bands; only the Rainier cherry (R) showed a reduced allergenic reactivity for bands at low molecular mass, while Napoleon (N) was very rich in LTP, so this extract was use to purified peach LTP. In all six cultivars, the allergen at 9 kDa was a LTP, as demonstrated by immunoblotting inhibition of the whole extract by purified Napoleon cherry LTP. With 40 μg the binding is completely inhibited, while a low level of radiostaining is detectable at 4 μg (**Figure 2**).

Many allergenic bands derive from cross-reactivity between cherry proteins and their homologues from birch pollen. In fact it can be noted, in **Figure 3**, the extremely different allergenic pattern of whole sample N, evaluated by individual sera from patients (nos. 1−7) having only an allergy to Prunoideae fruits (LTP-positive). These results confirmed those obtained by Pastorello et al. (*21*) showing that the allergenic profile of the patients with negative responses to birch pollen presented, apart from a very faint band around 30 kDa, only one strong IgE-binding component at 9 kDa, which was not so relevant in the immunological profiles of patients (nos. 8−12) with a simultaneous pollinosis.

In previous studies, chemical peeling was demonstrated to be very useful for removing LTP from peaches and apricots

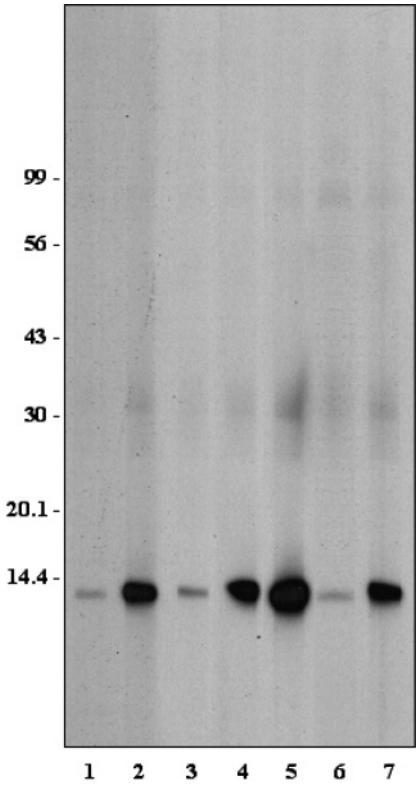

Figure 3. Immunoblotting of whole cherry N carried out with individual sera of LTP positive patients (1–7).

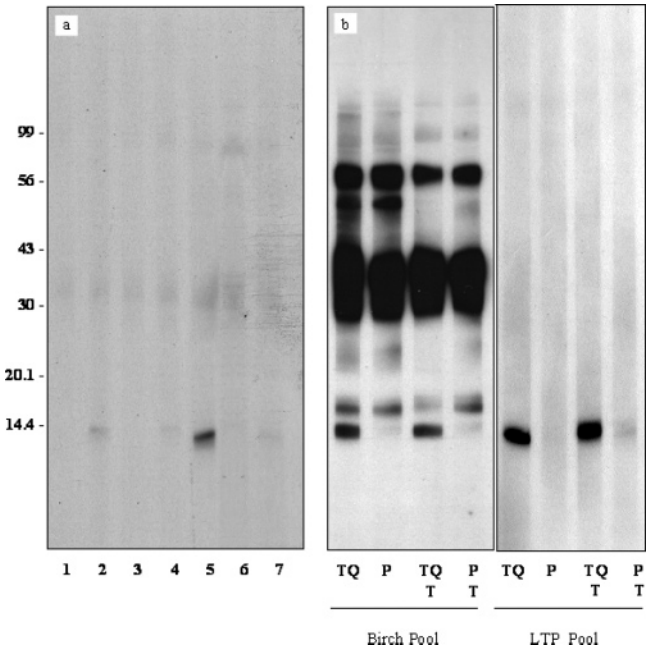

Figure 4. (**a**) Immunoblotting of chemically peeled cherry N, carried out with single sera of LTP-positive patients. (**b**) Immunoblotting of cherry N carried out with the birch pollen-sensitive (left) and LTP-positive pool sera (right). TQ, whole fresh cherry; P, chemically peeled; T, thermally treated.

(*13*, *22*). The importance of this finding was evident considering the high resistance of this allergen to other technological treatments, such as heating and proteolytic enzymes (*3*). Also in the case of cherry the chemical peeling demonstrated its effectiveness for this aim (**Figure 4a**); in fact almost all sera from LTP-positive patients did not show any IgE reactivity after fruit peeling. Only patient 5, the most reactive with whole fresh fruit (**Figure 3**), still had an IgE-binding band.

Considering thermal treatments, SDS−PAGE (not shown), and immunoblotting results did not show any significant reduction of allergenic proteins in all the samples considered, the major band around 30 kDa still kept its reactivity, as did the other allergens present in the native whole fruits. As an example, **Figure 4b** presents the immunological profile of whole fresh Napoleon cherry (TQ) and its peeled (P) and heated (T) derivatives. It can be also noted that the LTP band was not removed by the thermal treatment, as already demonstrated for peach (*3*).

For the industrial production of cherries in syrup, low anthocyanin content fruits, characterized by a light yellow or pink peel and flesh color and firm pulp, are generally preferred. Typically, the Durone della Marca cultivar is used, a cherry that has characteristics very close to some of our analyzed cherries, such as Napoleon and Rainier ones. For this reason, even if our results are related to Napoleon cherry, they can be assumed to be valid for all the cultivars analyzed.

The usual procedure starts with the immersion of fruits in an acidic solution containing $CaCl_2$ and $Na_2S_2O_5$, to maintain a certain consistency and to bleach fruits and preserve them. After about 2 weeks, a part of the cherries, whole and peeled, were submitted to protein extraction and analyzed by SDS−PAGE. Protein patterns were practically unchanged in comparison to their respective unprocessed cherries (data not shown), so we think that this immersion did not influence cherry allergenic profiles.

Protein extracts from whole and peeled cherries in syrup were analyzed by means of SDS−PAGE and immunoblotting. All the samples considered showed a noticeable reduction in their protein pattern; in fact, practically no band was evident by Coomassie staining. A commercial sample of cherries in syrup was analyzed under the same conditions, and also in this case SDS−PAGE did not show any significant protein band. However, a clear difference was revealed in the immunoblotting analysis, as shown in **Figure 5**, which reports results for cherry N, whole and peeled, and for the industrial sample.

These analyses, performed with the two pool sera considered in the present study, confirmed that the syruping process led to a drastic reduction of allergenic content. In particular, no sample shows a reactive band corresponding to LTP. Under the process conditions, consisting of several acidic washings, this allergen can be easily washed out, owing to its basic isoelectric point (above 9). The only still reactive band, which can be revealed by immunoblotting and with a molecular mass of about 14 kDa, was detectable by the birch-positive pooled sera (lanes a, **Figure 5**). This band was present in the whole sample (TQ-SY) and, with minor intensity, in the commercial cherries in syrup (COMM). It could be supposed that peel, still present in the commercial sample, hampered in some way the allergen diffusion, in particular if this protein corresponds to Pru av 4, which has a rather low p*I* (around 4.5). The minor intensity of this band, in the industrial sample, is probably due to fruit pitting before treatment, which leaves most of the peel and partially blocks the release of proteins present in the flesh.

Skin prick tests, performed with patients sensitized only to LTP (1−7), confirmed results of immunoblotting analyses (**Table 2**). Extracts from fresh whole cherry presented weal diameters similar among cultivars, while those deriving from peeled cherries showed a significant reduction ($p < 0.01$), with diameters less than or equal to the limit of 3 mm. Extracts of cherries in syrup, whole and chemically peeled, presented negative skin reactions.

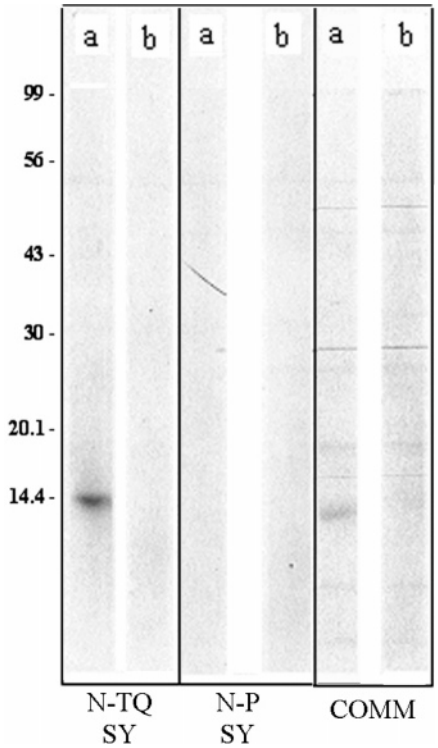

Figure 5. Immunoblotting of whole and chemically peeled cherry N in syrup and an industrial sample of cherries in syrup, carried out with birch-positive (lanes a) and LTP-positive pool sera (lanes b). TQ-SY, whole cherry in syrup; P-SY, chemically peeled cherry in syrup; COMM, industrial cherries in syrup.

Table 2. Results of Skin Prick Tests with Different Experimental Cherry Extracts, Performed with Patients Suffering Cherry Allergy Only, without Birch Pollinosis (LTP-Positive)[a]

sample	weal diameter (mm)	*t*-test
MV-TQ	3.9 (0.5)	**
MV-P	3.0 (0.6)	
<chgrow;lp;4q>GS-TQ	4.2 (0.5)	**
GS-P	3.0 (0.6)	
<chgrow;lp;4q>N-TQ	3.8 (0.9)	
N-P	neg	
N-TQ-SY	neg	
N-P-SY	neg	
<chgrow;lp;4q>histamine	5 (0.4)	

[a] MV, Mora di Vignola; GS, Grace Star; N, Napoleon. TQ, whole fresh cherry; P, chemically peeled; SY, cherry in syrup. neg, weal diameter is less than 3 mm. Standard deviation is presented in parentheses. **$p < 0.01$.

CONCLUSIONS

Our data, from in vitro and in vivo tests, can outline some relevant conclusions. All the protein extracts prepared from the whole cherry cultivars considered show a substantially similarity in the immunological pattern, weakening the possibility of finding a naturally hypoallergenic variety.

The use of different kinds of sera highlights that patients having an OAS syndrome not associated with birch pollinosis reacted with a unique band having a molecular mass of 9 kDa and corresponding to an LTP (immunoblotting inhibition with purified cherry LTP). As previously obtained for peaches and apricots (*3*, *22*), the chemical peeling confirms that LTP is confined in the cherry skin and could be effectively removed by this process. So it is possible to affirm that this treatment, if appropriately added in an industrial process, could lead to hypoallergenic derivatives, such as jams and fillings, suitable for people sensitized exclusively to LTP.

On the contrary, thermal treatment does not reduce significantly the allergenic profile of all samples and, in particular, is not able to remove LTP. Since many allergens responsible for birch cross-reactivity presented quite low thermostability (*9*, *23*), more severe heating could be evaluated in the future, without affecting the sensory quality of the final product and its industrial feasibility.

The syruping process very successfully removed allergenic proteins from samples, in particular when associated with chemical peeling, leading to anallergenic cherry derivatives. This process is very important for the two kinds of allergic patients considered because is an effective way of LTP removal, besides chemical peeling; furthermore, it removes almost all allergens pointed out by patients with a pre-existing pollinosis.

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