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Proteomic identification of allergenic seed proteins, napin and cruciferin, from cold-pressed rapeseed oils

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ABSTRACT

In Finland and France atopic children commonly react to seeds of oilseed rape and turnip rape in skin prick tests (SPT) and open food challenges. These seeds are not as such in dietary use and therefore the routes of sensitization are unknown. Possible allergens were extracted from commercial cold-pressed and refined rapeseed oils and identified by gel-based tandem nanoflow liquid chromatography mass spectrometry (LC–MS/MS). Napin (a 2S albumin), earlier identified as a major allergen in the seeds of oilseed rape and turnip rape, and cruciferin (an 11S globulin), a new potential seed allergen, were detected in cold-pressed oils, but not in refined oils. Pooled sera from five children sensitized or allergic to oilseed rape and turnip rape seeds reacted to these proteins from cold-pressed oil preparations and individual sera from five children reacted to these proteins extracted from the seeds when examined with IgE immunoblotting. Hence cold-pressed rapeseed oil might be one possible route of sensitization for these allergens.

1. Introduction

It has been shown that Finnish and French atopic children with clinical allergies to common foods frequently react to seeds of oilseed rape (*Brassica napus* ssp. *oleifera*) and turnip rape (*Brassica rapa* ssp. *oleifera*) in skin prick tests (SPT) and open food challenges (Poikonen et al., 2006, 2009). Major allergens (Bra n 1 and Bra r 1, respectively) were identified as 2S albumins; napins that are seed storage proteins in both of these oil plants (Puumalainen et al., 2006) and represent approximately 20% of the total protein. Napin, consisting of a small and large chain linked by disulphide bonds (Lonnerda & Janson, 1972), is reported to be extremely resistant to pepsin digestion and denaturation caused by heat and low pH (Murtagh et al., 2003). Thus it is possible that napin is not destroyed during conventional food processing. The main seed storage protein in these plants is an 11S globulin, cruciferin, two subunits that are composed of acidic and basic chains linked by a cysteine S–S bridge (Sjodahl, Rodin, & Rask, 1991). Cruciferin is one of the major allergens in white mustard and hazel nut (Beyer, Grishina, Bardina, Grishin, & Sampson, 2002; Palomares et al., 2005).

The route of sensitization in allergy to oilseed rape and turnip rape is unknown since the seeds as such are not in dietary use (Poikonen et al., 2006, 2008, 2009). Napins in oilseed rape, turnip rape, and mustard are highly cross-reactive (Poikonen et al., 2009; Puumalainen et al., 2006). In Finland, however, the consumption of mustard is minimal among lactating mothers or infants and therefore mustard is unlikely to be the first sensitizer. In contrast, in France, mustard is a common food allergen, and its consumption is the highest in Europe (Rance, 2003).

Mixed oil from oilseed rape and turnip rape (i.e. rapeseed oil) is the most widely used vegetable oil in Finnish households and food industry. The clinical relevance of allergy to oilseed rape and turnip rape is disputable, since there is no evidence that rapeseed oil, when ingested as such, causes or worsens symptoms in oilseed rape- and turnip rape-allergic patients (Poikonen et al., 2006). However, some vegetable oils, such as peanut oil, have been reported to contain allergenic proteins (Olszewski et al., 1998). Other vegetable oils have been reported to contain allergenic

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proteins although their role in allergic reactions, especially to refined oils, remains controversial (Crevel, Kerkhoff, & Koning, 2000; Morisset et al., 2003).

During the manufacture of cold-pressed oils, oil is pressed from the oilseeds without heating and the sediment from the seeds is settled down. Thus these kinds of oils may contain traces of seed proteins. To study whether commercial rapeseed oils carry seed allergens, we extracted possible proteins from the oils with acetone and analyzed them by tandem mass spectrometry (LC–MS/MS) from the stained protein gels. The allergenic potential of the oil and seed extracts was assessed by IgE immunoblotting using sera from children sensitized or allergic to the seeds of oilseed rape and turnip rape.

2. Methods

2.1. Acetone extraction of commercial rapeseed oils

Eight commercial rapeseed oils were selected from Finnish grocery stores. Four of them were cold-pressed and four refined (Table 1).

One volume of oil (30 ml) was extracted with four volumes of ice-cold acetone by incubating the mixture at −20 °C for 24–48 h. After centrifugation (4500 g/30 min/+4 °C) the precipitant was washed three times with four volumes of ice-cold acetone and re-suspended in 100 μl of Laemmli buffer (diluted from commercial 10× Tris Glycine SDS Buffer, Bio-Rad Laboratories) overnight at room temperature in a swinging shaker.

2.2. Electrophoresis and immunoblotting of oil protein fractions

The liquid phase from the re-suspended pellet was heated at 93 °C/3 min. We loaded acetone extractions (30 μl) onto 10 to 20% polyacrylamide gradient gels (Criterion™ Precast Gel, Bio-Rad Laboratories). Proteins were separated under reducing SDS–PAGE conditions and silver-stained with LC–MS/MS analysis optimized method (O'Connell & Stults, 1997). For a comparison, extracts from seeds of oilseed rape, turnip rape, and mustard were prepared and analyzed in SDS–PAGE as described earlier (Puumalainen et al., 2006) using PBS (Phosphate Buffered Saline) extraction.

For IgE-immunoblotting, proteins extracted from commercial oils were transferred after gel-electrophoresis to polyvinylidene difluoride (PVDF) membrane (Immobilon Transfer Membrane) and non-specific binding of antibodies was blocked with TBS-Tween®-20 detergent (0.05%), which does not interfere with the protein identification from the immunoblot by LC–MS/MS. Blotted membrane was incubated for 48 h with pooled sera (diluted 1:40) from five children (Patients 6–10 in Table 2, mean age 3.1 *y*, range 0.4–4.9 *y*) who were sensitized to oilseed rape and turnip rape seed proteins. Three of them were allergic to turnip rape, as confirmed by open food challenges. Biotinylated anti-human IgE (diluted 1:1000, Vector Laboratories Inc.) was added, followed by streptavidin-conjugated alkaline phosphatase (diluted 1:24,000, Zymed Laboratories Inc.) and substrate (Alkaline phosphatase conjugate substrate kit, Bio-Rad Laboratories). Dried Western blots were scanned with Image Scanner UMAX GTA 1100 Magic Scan v 4.6 (GE Healthcare). For the immunoblot of seed extracts (oilseed rape, turnip rape, and mustard) we incubated PVDF membrane overnight with individual sera (diluted 1:20) from five children (Patients 1–5 in Table 2, mean age 6.3 *y*, range 2.1–10.7 *y*) who were sensitized to oilseed rape and turnip rape seeds. Two of them were allergic to turnip rape in an open food challenge. The ethical committee of Tampere University Hospital approved the study, and informed written consent was obtained from the children's parents.

Table 1
Proteins detected on silver-stained SDS–PAGE and identified with LC–MS/MS from commercial rapeseed oils.

Oil number	Oil type, country of production	Detection SDS–PAGE + silver staining	Identification Q-TOF-MS	Protein UniProt accession number	Theoretical size kDa[a]	Mascot score[b]	No. matched peptides[c]/ sequence coverage[d]
1	Refined, Finland	<14 kDa	No proteins identified				
2	Refined, flavoured, Finland	<14 kDa	No proteins identified				
3	Refined, Belgium	Weak <14 kDa	No proteins identified				
4	Refined, Finland	Not detected	No proteins identified				
5	Cold-pressed, Finland	Multiple bands	Napin large chain	P27740	9.4	184	3/41%
				P09893	10.1	177	3/41%
			Cruciferin	P33523	30.6	292	6/24%
			Alpha chain	P33522	28.1	277	5/24%
				P33525	32.9	161	2/8%
			Beta chain	P33523	20.8	126	2/21%
				P33522	20.8	102	2/20%
6	Cold-pressed, Finland	Multiple bands	Napin large chain	P27740	9.4	270	5/41%
			Cruciferin	P33522	28.1	315	6/32%
			Alpha chain	P33523	30.6	202	4/20%
				P33525	32.9	163	3/13%
			Beta chain	P33523	20.8	132	2/9%
				P33522	20.8	121	2/9%
7	Cold-pressed, ecological, Finland	<14 kDa	No proteins identified				
8	Cold-pressed, Finland	Not detected	No proteins identified				

[a] Theoretical size of the cleaved subunits after maturation without possible post-translational modifications.
[b] Mascot score, $10 \times \log(P)$, where P is the probability that the observed match is a random event.
[c] No. matched peptides, number of peptides from digested gel spots that have sequence similarity with the identified protein.
[d] Sequence coverage, percentage of matching sequences with the whole protein sequence.

Table 2
Clinical characteristics of the patients whose serum was used in IgE-immunoblotting.

Patient	Total IgE (kU/L)	SPT oilseed rape (mm)	SPT turnip rape (mm)	Labial challenge to turnip rape[a]	SPT mustard (mm)	Other allergies or sensitization[b]	Asthma, sensitization to inhalant allergens
1	Not determined	Not determined	Not determined		Not determined	Potato	–
2	2350	5	8		10	Milk Wheat Egg	Pollen allergy
3	17,500	Not determined (RAST 5.1 kU/L)	Not determined		7	Milk[c] Wheat Egg	Pollen allergy
4	6200	5	13	+	7	Milk Wheat Egg	Asthma, pollen allergy
5	34,600	7	5	+	6	Milk Wheat Egg	Asthma, pollen allergy
6	2050	7	9	+	10	Milk[c] Wheat Egg	Asthma, pollen allergy
7	132	11	8		20	Milk Wheat Egg	Asthma, pollen allergy
8	1510	7	6	+	6	Wheat Egg	Asthma, pollen allergy
9	727	6	15		15	Milk Wheat Egg	–
10	3820	10	13	+	5	Milk Wheat Egg	Asthma, pollen allergy

[a] Labial challenge to crushed seed of turnip rape as described by Poikonen et al. (2006, 2009); Patients 1, 2, 3, 7, and 9 were not tested.
[b] Sensitization confirmed by skin prick test or ImmunoCAP (specific IgE, Phadia Ab).
[c] Other food allergy confirmed by open food challenge.

2.3. Protein identification from gels and immunoblot membranes

Silver-stained protein spots were cut out from the gels, reduced with dithiothreitol and alkylated with iodoacetamide before in-gel digestion with trypsin (Promega) for 16 h at 37 °C (Korolainen et al., 2006). Briefly, after removal of the supernatants to fresh tubes, we extracted the remaining peptides in gel pieces twice with 100 μl of 5% formic acid in 50% acetonitrile. Protein bands excised from the immunoblotted membrane were treated similarly after the stripping of antibodies from the membrane with 0.2 M NaOH (Suck & Krupinska, 1996). Combined peptide extracts were dried in a vacuum centrifuge. We then analyzed each peptide mixture by automated nanoflow capillary LC–MS/MS, using a CapLC system (Waters) coupled with an electron spray ionization quadrupole time-of-flight mass spectrometer (Q-TOF Global, Waters). The reversed phase separation of peptides was carried out using a 75 μm × 15 cm NanoEase Atlantis® dC_{18} column (Waters) at 250 nl/min flow rate. Solvent A was 0.1% formic acid in 5% ACN, and solvent B was 0.1% formic acid in 95% ACN. Peptides were eluted from the column with a linear gradient of 0–60% solvent B in 30 min. The obtained mass fragment spectra were searched for in the NCBInr database, using the in-house Mascot v.2.1 (Matrix Science Ltd.) with the following parameters: trypsin specificity; one missed cleavage; precursor ion mass accuracy of 0.2 Da and 0.5 Da mass tolerance for fragment ions; fixed modification was carbamidomethylation of cysteine and oxidation of methionine, histidine and tryptophan were accepted as variable modification.

2.4. Estimation of protein concentration in oil extracts

As the precipitated extracted proteins were dissolved in Laemmli buffer, common protein quantification kits could not be applied to determine protein concentrations in the samples. We estimated the amount of proteins from the spot intensities of both the silver- (Fig. 1A) and SYPRO Ruby- (Molecular Probes) stained SDS–PAGE gels (Lopez et al., 2000), because the SYPRO Ruby dye yields a better linear quantification than silver-staining. The intensities of gel spots were calculated using the ImageQuant 5.2 programme (GE Healthcare) and compared to the known spot.

3. Results

Possible protein bands from acetone-extracted commercial rapeseed oils in silver-stained SDS–PAGE gels were visible in all tested oils except for one of the refined oils and one of the cold-pressed oils (Fig. 1A and Table 1). LC–MS/MS analysis of the gel bands identified napin (2S albumin) large chain and cruciferin (11S globulin) α and β chains from two of the four commercial cold-pressed oils. Several different protein sequences have been registered for rapeseed cruciferin (e.g. UniProt accession numbers P11090, P33522, P33523, P33524, and P33525). The homology between isoforms varied from 60% to 99%. We identified subunits of three of these isoforms from the acetone extracted oils (Fig. 1A and Table 1). Because their molecular weights are close to each other they may appear on the gel as diffused bands. There are scarce reports of post-translational modifications either in napin or cruciferin molecules (Jolivet et al., 2009). Excised gel spots from refined oils remained unidentified (Fig. 1A and Table 1). The failure to identify gel spots in LC–MS/MS could be due to very low protein concentration, the absence of peptides of suitable size, or the silver staining produced by something other than proteins, for example lipids.

We estimated the allergen contents of the two tested oils to evaluate their potential to induce sensitization or clinical symptoms in allergic patients. In cold-pressed oils, the amounts of napin and cruciferin were 5–19 μg/l and 7–27 μg/l (lower values from

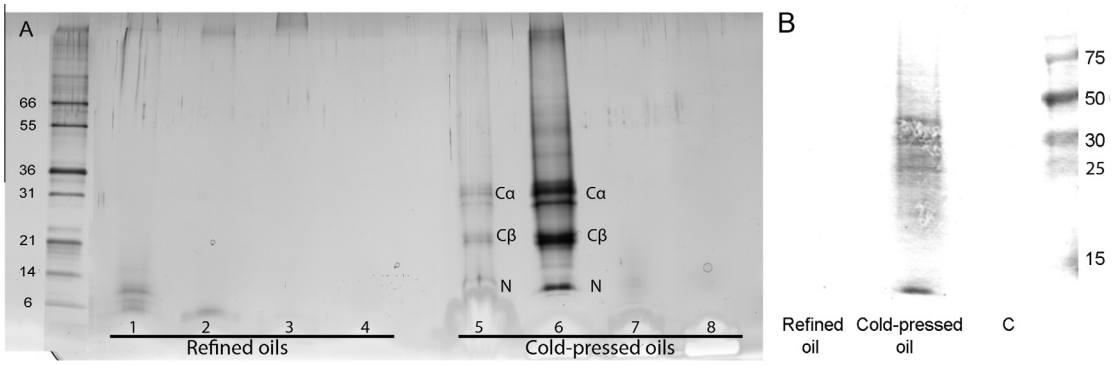

Fig. 1. (A) Silver-stained SDS–PAGE of four acetone-extracted commercial cold-pressed and four refined rapeseed oils (numbering as in Table 1). Cα = cruciferin α chain, Cβ = cruciferin β chain, N = napin large chain. Molecular weight markers (Mark12, Invitrogen) are presented on the left (kDa). (B) IgE-immunoblot analysis of PBS-extracted refined and cold-pressed rapeseed oils (oil numbers 4 and 5 in (A) with pooled sera from five oilseed and turnip rape-sensitized or allergic children (Patients 6–10 in Table 2). Molecular weight markers (Rainbow, GE Healthcare) are presented on the right side. C = serum from a non-allergic adult control.

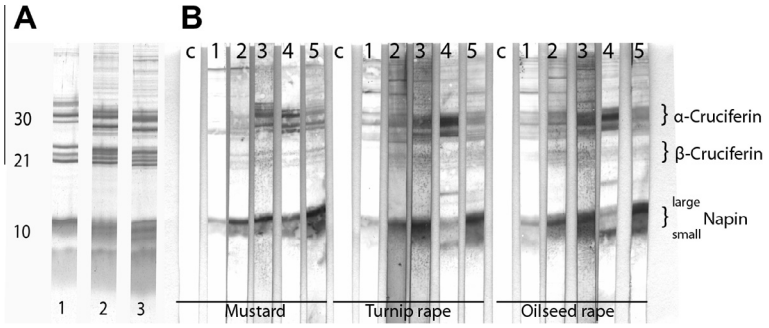

Fig. 2. (A) Coomassie blue -stained SDS-PAGE of seed extracts of mustard (lane 1), turnip rape (lane 2) and oilseed rape (lane 3). (B) IgE-immunoblot analysis to seed extracts with serum from five sensitized or allergic children (Patients 1–5 in Table 2). C = serum from a non-allergic adult control.

silver-stained and higher from SYPRO Ruby-stained gels). Consequently, the estimated concentration of allergens together in the acetone extraction was 12–46 μg/l.

Protein extracts from commercial rapeseed oils were IgE-immunoblotted (Fig. 1B) using pooled sera from five children with positive SPTs to turnip rape. In cold-pressed oils, we observed reactivity around protein sizes of <15 kDa and between 20 and 35 kDa, which agree with the expected molecular masses of napin (9 and 4 kDa) and cruciferin (20 and 30 kDa) subunits. Nothing was detected from the refined oil (Fig. 1B).

The allergenic potential of cruciferin is shown here with PBS-extracted seed extracts from oilseed rape, turnip rape, and mustard, which are known to contain napin and cruciferin allergens (Menendez-Arias, Moneo, Dominguez, & Rodriguez, 1988; Palomares et al., 2005) by analysis with SDS–PAGE and IgE immunoblotting, using five children with positive SPT results to turnip rape (Fig. 2). The same protein sizes as above presented major reactivity (Fig. 2). For identification, these seed extract bands from the gel (Fig. 2A) as well as directly from the seed-extracted IgE immunoblot (Fig. 2B) were analyzed by LC–MS/MS and were confirmed to be napin and cruciferin. Because no other proteins came up from the chosen gel and membrane spots, the observed immunogenicity must be due to cruciferin and napin.

4. Discussion

We found napin (2S albumin) and cruciferin (11S globulin) in two of the four tested commercial cold-pressed rapeseed oils. Both of these proteins were also recognized immunologically by patients sensitized or allergic to seeds from oilseed and turnip rapes, indicating their potential allergenicity.

Cruciferins and napins constitute approximately 60% and 20% of the mature seed proteins, respectively (Hoglund, Rodin, Larsson, & Rask, 1992). They share homology in protein structure, and many of the polyclonal antibodies detect both of them (Jolivet et al., 2009). Due to their high abundance, cruciferins should be regarded equally important allergens in oilseed rape and turnip rape as napins.

The protein content of vegetable oils has not been studied systematically in allergology. In the acetone extracted cold-pressed oil, the amount of napin and cruciferin together was 12–46 μg/l. This concentration range is roughly similar to the reported total protein concentrations in previous studies with different vegetable oils, extraction methods and protein measurements (Olszewski et al., 1998; Paschke, Zunker, Wigotzki, & Steinhart, 2001). The actual protein concentrations in rapeseed oils are higher, as some proteins, such as oleosin (Leduc et al., 2006), were probably lost already during the extraction. Oleosins are amphipathic in character and surround seed oil bodies, which are intracellular lipid-storing particles (Jolivet et al., 2009). Therefore part of the precipitated proteins stayed water insoluble and were excluded from the estimation of the protein amount.

The recommended daily consumption of rapeseed oils (30 ml, about two tablespoons per day, ("US FDA/CFSAN Qualified Health Claims: Letter of Enforcement Discretion Unsaturated Fatty Acids from Canola Oil and Reduced Risk of Coronary Heart Disease," 2011) contains 360–1380 ng of protein. Although the amount of protein in vegetable oils seems to be minute, allergic reactions even to refined oils have been reported (Morisset et al., 2003).

The crucial question is how much of the relevant allergen is needed to provoke an allergic reaction in a sensitized individual. Thresholds of reactivity differ between allergens, and it has been suggested that the reaction level for oil protein allergens may be much lower than for conventional allergens (Morisset et al., 2003).

Despite the present results, we suggest that restriction of the use of rapeseed oil is not required for atopic individuals who are not sensitized or allergic to the seeds of oilseed and turnip rape. Rapeseed oils have ideal fatty acid content, and have beneficial health effects on serum lipids and lipoproteins (Gulesserian & Widhalm, 2002). On the other hand, the possibility cannot be excluded that allergic patients sensitized to seeds of oilseed rape and turnip rape might suffer symptoms from oil made from these same plants. Refined oils are more purified and usually contain less protein than cold-pressed oils and could be recommended for oilseed rape- and turnip rape-allergic individuals. Thus far no clinical evidence of allergic reactions after ingesting rapeseed oil has been reported. As most of the studies concerning the allergenic potential of other vegetable oils are clinical reports of individual cases, estimating the risk to the population is not possible.

In conclusion, earlier identified allergenic napins (2S albumin) and new potential allergenic cruciferins (11S globulin) can be detected in cold-pressed rapeseed oils with sensitive proteomic methods. These oils might be one possible route of sensitization for these seed allergens. However, evaluation of the clinical importance of the exposure to low concentration allergens in rapeseed oil needs further investigation in allergic individuals.

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