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Characterization of *Cannabis sativa* allergens

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marijuana; allergen; cannabis; type I hypersensitivity

INTRODUCTION

Cannabis sativa is an herbaceous angiosperm belonging to the family Cannabaceae. *C. sativa* and its resinous derivative hashish, has a variety of industrial, and more recently medicinal applications. The plant is most well-known for its use as a recreational drug as it contains the psychoactive compound, delta-9-tetrahydrocannabinol (Δ^9 -THC). An increase in marijuana consumption has been observed among teenagers in the United States.¹ As a result of the increasing social and medical usage, reports of allergic sensitization to marijuana are increasing in the peer reviewed literature.²⁻¹⁰

Cases have been reported where hypersensitivity and even anaphylactic responses have been associated with marijuana use and clinical symptoms include sore throat, nasal congestion, rhinitis, pharyngitis, wheezing, dyspnea, angioedema and lacrimation.^{2-4, 11, 12} In chronic and high dose users more severe manifestations of bronchitis and asthma with reduced vital capacity have been reported.¹²

Allergic sensitization to *C. sativa* has been reported in occupational settings as well.¹³ Hemp workers involved in processing hemp fibers at a textile mill had significantly higher

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prevalence of chronic respiratory symptoms, attributed to byssinosis. Sensitization of laboratory workers that handle and test marijuana has also been reported.¹⁴⁻¹⁶

The allergens of *C. sativa* and its various derivatives are poorly characterized. Although Δ^9 -THC has been suggested to be an allergen¹¹, more recent studies show type I hypersensitivity to high molecular weight proteins derived from *C. sativa*.^{4, 6, 9, 10, 17, 18} In a recent study in Spain, IgE binding proteins were observed with molecular weights ranging from 10 – 69 kDa.⁴ Others have identified patients sensitized to two prominent IgE binding bands located at 10 and 14 kDa⁹ and a 9 kDa lipid transfer protein was identified to bind IgE from a patient sensitized to *C. sativa*.¹⁷ However, the identity of most allergens from *C. sativa* remains unknown and no allergens are currently listed by the International Union of Immunological Societies (IUIS) allergen nomenclature sub-committee.

Previously, we reported 17 individuals that were skin prick test (SPT) positive to crude extracts of marijuana buds and flowers.³ In all patients, exposure was primarily through smoking and direct contact with the plant. However, one patient was additionally exposed through consumption of marijuana tea. In this study, we characterize patient IgE reactivity to root, leaf, flower, and bud extracts in an attempt to identify potential allergens for patients sensitized to *C. sativa*.

METHODS

Patient population

Sera were obtained from 17 individuals with inhalation and contact symptoms that were SPT positive to a crude *C. sativa* extract from buds and flowers (macerated in water for 15 min), as previously reported.³ The most common symptoms following exposure to marijuana included rhinitis and conjunctivitis, periorbital angioedema, wheezing and contact urticaria. Most SPT positive patients had primary exposure to *C. sativa* either through smoking and direct contact to the plant. One patient also consumed marijuana tea, which resulted in gastric cramping, vomiting and anaphylaxis. Exposure was due to recreational use of marijuana in all cases. Six additional patients with similar clinical presentation and positive SPT to *C. sativa* were also recruited during the study. Additionally, serum from seven individuals that consumed *C. sativa* but were SPT negative to *C. sativa* extracts and serum from a laboratory worker with no known exposure to *C. sativa* were used as negative controls. De-identified sera were sent to the National Institute for Occupational Safety and Health (NIOSH) for serological analysis of IgE binding protein and proteomic analysis. Written consent was obtained from human subjects and the ethics approval was obtained for this study from the Canadian Shield Ethics Review Board.

Cannabis sativa extracts

For protein analysis, extracts from various parts of *C. sativa* L. Mexican variant. (leaf, root, female flowers, and buds) were prepared at the National Center for Natural Product Research, Research Institute of Pharmaceutical Sciences, School of Pharmacy at the University of Mississippi. Un-pollinated female flowers were used for generation of extracts and were devoid of seed components. Proteins were extracted from 100 mg of root, leaves, buds, and flowers using the Total Plant Protein Extraction Kit (Sigma), according to manufacturer's instructions.

SDS-PAGE, 2-D gel electrophoresis and Western blot analysis

Individual *C. sativa* protein extracts (25 μ g) were separated by SDS-PAGE using 12% polyacrylamide gels and stained with Imperial™ Blue (Thermo Scientific, Rockford, IL) according to manufacturer's instructions.

For Western blot analyses, 25 µg of individual *C. sativa* protein extracts were separated as described above and transferred overnight to a nitrocellulose membrane (0.22 µm, BioRad). The membrane was blocked using Tris-buffered saline (TBS) containing 3% bovine serum albumin (blocking buffer) for 1 h. The membrane was incubated with pooled sera from SPT-positive patients, diluted 1:5 (v/v) in blocking buffer for analyzing patient reactivity to the extracts from different parts of *C. sativa* plants. For individual patient screening, the membrane was transferred to a BIO-RAD Multi Screen apparatus (BioRad, Hercules, CA) for analysis and incubated with either individual SPT-positive, or individual SPT-negative patient sera diluted 1:5 (v/v) in blocking buffer and incubated overnight at 4°C on a rocker. The following morning, the membrane was washed and incubated with mouse anti-human IgE monoclonal antibody (clone GE-1) (Sigma) diluted 1:5000 v/v in blocking buffer for 2 h on a rocker at room temperature (RT). The membrane was washed three times with TBS containing 0.05% Tween-20 v/v (TBST) and incubated with alkaline phosphatase conjugated goat anti-mouse IgG antibody (H+L) (Promega, Madison, WI) diluted 1:5000 v/v in blocking buffer for 1 h on a rocker at RT. The membrane was incubated with 1-Step NBT/BCIP (Promega) substrate solution for 15-20 min. The reaction was stopped by washing the membrane with distilled water.

To investigate the role of glycosylation in IgE reactivity to *C. sativa* proteins, extracts from roots, leaves, buds and flowers were partially deglycosylated as previously described.¹⁹ The extracts were first separated by SDS-PAGE and transferred to nitrocellulose membranes, each membrane containing samples from the four different plant sources. One membrane was treated with 50 mM sodium periodate in 50 mM acetate buffer for 1 hr in dark at RT while the other membrane served as a control and was treated with 50 mM acetate buffer alone for the same duration. The membranes were washed 3 times for 10 mins with phosphate buffered saline pH 7.4 (PBS) containing 0.05% Tween 20 (v/v) (PBST) and blocked in PBST containing 3% non-fat dry milk (PBST-M). The membranes were then incubated with pooled serum from SPT-positive patients, diluted 1:5 (v/v) in PBST-M and processed for determination of IgE reactivity as described above.

For 2-D gel electrophoresis, *C. sativa* protein extracts of leaves were processed as described earlier. Briefly, proteins were solubilized in rehydration solution containing 8 M urea, 20 mM dithiothreitol (DTT), 2% CHAPS, 0.5% IPG buffer and bromophenol blue. The solution was loaded onto a 7 cm IPG strip pH 3-10 (GE Healthcare, Uppsala, Sweden) and focused using an IPGphor apparatus (GE Healthcare). The strip was equilibrated in 50 mM Tris-HCl pH 8.8 with 6 M urea, 65 mM DTT, 30% glycerol, 2% SDS and bromophenol blue prior to separation of focused proteins in the 2nd dimension using SDS-PAGE. Strips were processed in duplicate for each extract sample and one set of gels was stained with ImperialTM blue protein stain while the other was processed for Western blot analysis.

All Western blots were analyzed with secondary and tertiary antibody control, by incubating samples with mouse anti-human IgE antibody and goat anti-mouse IgG (H+L) and developing with NBT/BCIP as described earlier.

Proteomic analysis and homology searching

Protein spots of interest were excised from 2D SDS-PAGE gels. Spots were destained, reduced, alkylated, and digested with porcine trypsin (Sigma-Aldrich, St. Louis, MO) according to the methods of Shevchenko et al.²⁰ Reduction was performed with 20 mM tributylphosphine (Sigma) and trypsin digestion was performed overnight at 37°C with shaking (500 rpm). Digested supernatant was combined with extraction buffer and concentrated in a centrifugal evaporator (Savant DNA120, Thermo Scientific, Asheville, NC) prior to mass spectrometry analysis.

Aliquots of individual digested protein spots were subjected to ultra-performance liquid chromatography (UPLC) on a nanoACQUITY system (Waters, Milford, MA). Samples were desalted on a 180 $\mu\text{m} \times 20\text{ mm}$ Symmetry C₁₈ (5 μm particle) trap column with 100% solvent A (0.1% formic acid in distilled, deionized water). Analytical separation was performed on a BEH130 C₁₈ (1.7 μm particle) using a gradient of 97/3 A/B (0.1% formic acid in acetonitrile) to 50/50 A/B.

Eluent from the nanoUPLC system was directed to the positive nanoelectrospray (+nanoESI) source of a Synapt (Waters) quadrupole time-of-flight mass spectrometer (qTOF MS). Dry nitrogen was supplied as a desolvation gas. Ultra-high purity argon was utilized as the collision gas for collision-induced dissociation (CID). Data were acquired in a data-dependent fashion according to the following criteria: MS survey scans were performed from 100 to 1500 u over 1 second. A mass-to-charge ratio (m/z) of interest was selected for tandem mass spectrometry (MS/MS) if it met the intensity threshold of 20 counts per second and was either doubly or triply charged, as determined by examination of the m/z gap between isotopes.

Data were analyzed by using ProteinLynx Global Server v 2.4 to search the entire non-redundant SwissProt protein database (www.uniprot.org). The search was constrained by using 100 ppm mass accuracy ($m/\Delta m$), carbamidomethyl cysteine as a fixed modification, trypsin as the enzyme, and a minimum of 2 peptides to match. The entire non-redundant database was searched for the full dataset to eliminate any false positive matches from common in gel digest contaminants such as keratin or trypsin auto digestion products. Unmatched *de novo* peptide sequences were blasted against SwissProt using the PAM30MS scoring matrix.

The *de novo* peptide sequences were further analyzed to identify putative allergens. The translated nucleotide database or Transcriptome Shotgun Assembly (TSA) of *Cannabis sativa* (taxid: 3483) was used for identification of putative mRNA sequences that transcribe the *de novo* peptide sequences. The TSA database for *C. sativa* was recently published in NCBI database collection with accession numbers between GenBank numbers JP449145 and JP482359.²¹ Putative mRNA sequences were translated using EMBOSS Transeq software and appropriate in-frame translated protein sequences were identified in BLAST searches for homologous proteins.

RESULTS

IgE reactivity to various parts of *C. sativa* plant

The protein profiles of root, leaf, bud, and flower extracts of *C. sativa* are demonstrated in Fig. 1A. Protein profiles of *C. sativa* leaves, buds, and to some extent flowers were similar with a prominent ~50 kDa band common to these extracts. The extracts from flowers demonstrated prominent protein bands at ~18 and ~35 kDa that were absent in the other extracts. In contrast, the protein profile of *C. sativa* root extract was distinct, but was lacking the predominant bands present in the other extracts.

For extracts from each of the *C. sativa* plant sources, IgE from pooled serum reacted to multiple protein bands ranging between 20–100 kDa. Immunoblotting showed some variability in IgE binding to each plant component (Fig. 1B); however, IgE reactivity was largely similar for extracts collected from leaves, buds, and flowers. Prominent staining was observed at ~50 kDa and 23 kDa in these extracts. The IgE reactivity of pooled sera to root extract showed a profile distinct to extracts from the other sources, with prominent staining at ~35 kDa. In addition to these prominent bands, immunostaining was also observed for multiple other bands as well.

Individual patients had a wide spectrum of IgE reactivity ranging from 10 to 100 kDa, with considerable variability. SPT positive patients 8/23 (35%) demonstrated a similar pattern of IgE reactivity (Fig. 2A). In leaf extracts, 13/23 patients (56.5%) reacted to the ~50 kDa protein band. Eight patients (P3, P8, P16-P21) showed immunoreactivity to a band localized at ~23 kDa. Minor bands associated with IgE binding were also localized at ~15 kDa, ~35 kDa and ~75 kDa.

With the root extract, 9 patients (39%) did not show IgE binding to root extract proteins (Fig. 2B). Of the remaining 14 patients, the reactivity was variable and mostly restricted to a 37 kDa band. Minor bands were identified at ~10 kDa, 13 kDa, 15 kDa, 17 kDa, 25 kDa 50 kDa, and 100 kDa. The 8 patients with the strongest reactivity to the root extracts were also the individuals exhibiting the strongest reactivity to the leaf proteins (P3, P8, P16-P21).

The data also demonstrates considerable variability in IgE reactivity between the SPT positive patients. Patients that demonstrated SPT reactivity to *C. sativa* extracts during clinical examination did not necessarily exhibit IgE reactivity to *C. sativa* extracts in Western blot analysis (Fig. 2A and 2B). Furthermore, some SPT negative patients demonstrated IgE reactivity to *C. sativa* extracts especially from leaves. This may be attributed to cross-reactive allergens present in plants as we did not observe any immune reactivity with the reagent antibody controls (data not shown). It is conceivable that immunoreactivity may be towards microbial contaminants in the resource materials used for testing. Although no testing was performed to assess the microbial burden of these samples, it is noted that most of these patients were SPT negative to *Aspergillus*.³

IgE reactivity after deglycosylation

In order to assess the IgE reactivity to *C. sativa* extracts in absence of glycosylated epitopes, the sodium periodate-treated membranes were analyzed using Western blot analysis (Fig. 3). Many protein bands with positive IgE reactivity in lanes 1-4, were not reactive on membranes treated with sodium periodate lanes 5-8. IgE reactive to deglycosylated protein bands were observed in all extracts of *C. sativa*. For roots, the predominant immunoreactive bands were observed at 70 kDa, 50 kDa, 35 kDa and 27 kDa. In leaves, a prominent band was observed at ~50 kDa along with bands at 35 kDa, 25 kDa and at 23 kDa. The IgE reactivity was very similar for extracts from buds and flowers. In addition to IgE reactivity at 50 kDa, the extracts showed reactivity to bands at 70 kDa and ~45 kDa with the 45 kDa appearing to increase in intensity with deglycosylation. Some other minor bands with IgE reactivity were also observed.

2D gel electrophoresis and proteomic analysis

2D gel electrophoresis was combined with Western blotting to select *C. sativa* bands for allergen identification. 2D immunoblot demonstrated IgE reactivity at ~50 kDa to a large area along the pH gradient (3-10) (Fig. 4). A number of peptides could be identified by proteomic analysis for spots 1-4 including the heavy chain subunit of Ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) and peptides for ATP synthase (spots 1 and 2) (Supplementary Table 1). IgE binding to the 2D blot appeared the most intense to a series of spots around 23 kDa. Peptide sequences were obtained for these spots (6-10), and identified as oxygen-evolving enhancer protein 2 a 23 kDa protein belonging to the photosystem II. Spot 5 (~45 kDa) which increased in IgE binding intensity with deglycosylation contained peptides from several enzymes including RuBisCO activase 1 & 2, glutamine synthetase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase and camphor resistance protein. In the root extract, we identified ATP synthase beta subunit and luminal binding protein as potential allergens (data not shown).

DISCUSSION

Cannabis sativa is widely used for various medicinal and industrial purposes.²² Recently, an increase in the recreational use of *C. sativa* has been reported.^{1, 23} Studies have focused on the overall health effects of *C. sativa* consumption with less attention to aspects such as respiratory morbidity or allergic sensitization.²² Allergic responses to *C. sativa* are rarely reported; however, it is likely that the social and legal aspects surrounding the recreational use of marijuana may discourage patients from addressing their symptoms with a physician. Exposure to *C. sativa* has been reported to sensitize individuals and even results in serious anaphylactic reactions.^{3, 11} Most cases described in the literature involve sensitization to *C. sativa* via multiple exposures presumably involving respiratory, cutaneous, and/or oral routes.^{6, 24} Allergic sensitization to *C. sativa* has also been attributable to environmental *C. sativa* pollen exposures, particularly in the southwestern region of United States.²⁵ To date, the allergens associated with *C. sativa* exposure and sensitization have remained relatively uncharacterized.

In the present study, IgE binding to *C. sativa* root, leaf, flower, and bud extracts was determined. The protein and IgE binding profiles observed in leaf, flower, or bud extracts were similar indicating some common proteins in these parts of the plant. As expected, the protein profile and the IgE reactivity of the root extract was somewhat different. Analysis of individual SPT patient IgE reactivity demonstrated a variability in patterns of reactivity, consistent with a previous observation⁹. Some patients that were determined to exhibit a positive SPT result did not show evidence of IgE binding in Western blot analysis (Fig. 2).

In the leaf extract, the predominant IgE reactivity was observed at 50, and 23 kDa, even after deglycosylation. Using proteomic analysis, the 50 kDa protein was identified as the larger chain subunit of RuBisCO and the ~23 kDa protein was identified as oxygen-evolving enhancer protein 2 of the photosystem II, while multiple peptides and proteins were identified for 45 kDa protein. Previous studies have attempted to identify allergens associated with *C. sativa* sensitization^{9, 17} and sensitization to a lipid transfer protein (LTP) was determined in one case report.¹⁷ A 10 kDa protein band, which may correspond to LTP, was proposed in European and Asian cohorts as a potential *C. sativa* allergen.^{2, 9, 26} Although, LTPs are common sensitizers in foods and plant derived aerosols,^{27, 28} no LTP sequences were identified in our LCMS analysis; however, IgE reactivity was observed at ~10 kDa for 2 patients with the *C. sativa* root extract.

The present study follows previous reports of clinical determination of hypersensitivity to *C. sativa*.^{3, 5} In this study, the prominent IgE reactive bands were identified as RuBisCO and oxygen-evolving enhancer protein 2. Multiple peptides for the large (55 kDa) and small chains (14 kDa) of RuBisCO were identified in the LCMS analysis. RuBisCO is the most abundant protein in nature and catalyzes the conversion of D-ribulose 1,5-bisphosphate to 2,3-phospho-D-glycerate in the presence of carbon dioxide, a rate-limiting step for photosynthesis.²⁹⁻³¹ Although RuBisCO is generally susceptible to rapid degradation by gastric fluid,^{32, 33} IgE responses have been generated upon parenteral administration of this protein.^{34, 35} Presumably, the patients in the present study were sensitized by inhalation or dermal contact with dried marijuana, potentially bypassing degradation by gastric fluid. Recently, several reports have suggested that RuBisCO can be an allergen in other plants.³⁶⁻³⁸

Another IgE binding protein identified in this study was ATP synthase. In one study, sequence homology was demonstrated between a bovine allergen and one of the subunits of ATP synthase which forms the basis for a potential cross-reactivity.³⁹ Glyceraldehyde-3-phosphate dehydrogenase was also identified as one of the putative allergens of *C. sativa* in

our studies. This protein has been identified as a major allergen of *Triticum aestivum* (wheat), several fungi, and as a major allergen in rambutan (*Nephelium lappaceum*)-induced anaphylaxis.⁴⁰⁻⁴² Several other proteins identified in this analysis have also been reported as mold or pollen allergens in previous studies. Phosphoglycerate kinase is a characterized allergen of *Candida albicans*.⁴³ Luminal binding protein (BiP) is a highly conserved hazel pollen allergen (Cor a 10), which functions as a chaperone during protein synthesis, and is reported to be a cross-reactive pollen allergen.⁴⁴ BiP also shares a high degree of sequence homology to heat shock protein 70 (Hsp70), which has been reported as an allergen in many fungi.^{45, 46} Oxygen evolving enhancer protein was a prominent allergen in our analysis; however, there are no studies in the literature that currently report this protein to be an allergen.

Analysis of IgE reactivity on sodium periodate-treated membranes demonstrated an IgE response to carbohydrate determinants. It is possible that patients who tested positive during skin prick test analysis may have some IgE towards the glycosylated moieties on proteins as demonstrated by the Western blot analysis. To the best of our knowledge, no previous studies have examined this possibility in *C. sativa* sensitization and our studies suggest that some of the reactivity could be due to cross-reactivity carbohydrate determinants. Further analysis is required to determine the identity and clinical relevance of these IgE binding epitopes. It is not known if these IgE reactive oligosaccharides are similar to other cross-reactive carbohydrate determinants that are monovalent and while they exhibit extensive cross-reactivity during analysis of plant extracts *in vitro* they have limited activity in SPT.⁴⁷ Due to limitations in the amount of samples, the cross-reactivity to carbohydrates could not be further addressed in the current study.

In this study, sensitization to *C. sativa* allergens has been characterized and peptides from enzymes associated with the primary metabolism of plants, such as RuBisCO, oxygen evolving-enhancer protein, ATP synthase, phosphoglycerate kinase, and glyceraldehyde-3-phosphate dehydrogenase were identified as allergens. We also determined that IgE reactivity to carbohydrates also exists. Due to the growing use of marijuana as a recreational drug and as an antiemetic in patients receiving chemotherapy, allergic sensitization to *C. sativa* is expected to increase. Further research is needed to understand the variability in underlying immunological mechanisms and development of standardized reagents for clinical and diagnostic testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. SDS-PAGE showing *C. sativa* protein extracts profiles and pooled-serum IgE reactivity Protein extracts were derived from leaves, buds, flowers and roots of *C. sativa*. **A.** Imperial™ protein stained SDS-PAGE gel. **B.** Pooled patient serum IgE reactivity. Molecular weight (MW) in daltons is indicated in the left margin as estimated using Precision plus protein™ All blue standards.

Fig. 2. IgE reactivity to *C. sativa* protein extracts

A. leaves, B. roots. N1: Healthy donor from the laboratory, N2-N7 sera from SPT-negative patients, P1-P23 were sera from *C. sativa* SPT-positive patients. MW-Precision plus protein™ All blue standard.

Fig. 3. Effect of sodium periodate on IgE reactivity

Protein extracts separated by SDS-PAGE were transferred to nitrocellulose membranes. Lanes 1 and 5 (roots), Lanes 2 and 6 (leaves), Lane 3 and 7 (buds) and Lanes 4 and 8 (flowers), represent various extracts. Lanes 1-4 were treated with 50 mM sodium acetate as a control, and lanes 5-8 were treated with 50 mM sodium periodate in 50 mM sodium acetate. MW indicated in the left margin was determined using Precision plus protein™ All blue standards.

Fig. 4. Identification of IgE-binding antigens

2D-Western blot analysis of IgE-reactivity to *C. sativa* leaf extracts using pooled serum IgE from SPT-positive patients. Proteomic analysis was performed by *de novo* sequencing of the peptides and matched with the existing UniProt database. MW-Precision plus protein™ All blue standard.