

Molecular Characterization of Major Cat Allergen Fel d 1

EXPRESSION OF HETERODIMER BY USE OF A BACULOVIRUS EXPRESSION SYSTEM*

Received for publication, September 16, 2004, and in revised form, November 15, 2004
Published, JBC Papers in Press, November 15, 2004, DOI 10.1074/jbc.M410668200

Ulla Seppälä‡, Per Hägglund§¶, Peter A Wurtzen‡, Henrik Ipsen‡, Peter Thorsted‡,
Thomas Lenhard‡, Peter Roepstorff§, and Michael D. Spangfort‡||

From the ‡Departments of Vaccine Research and Experimental Immunology, ALK-Abelló, 2970 Hørsholm, Denmark and
the §Department of Biochemistry and Molecular Biology, University of Southern Denmark, 5230 Odense, Denmark

Fel d 1 is a major cat allergen inducing allergic rhinitis and asthma in sensitized individuals. It has a more complex structure when compared with other allergens and therefore expression of recombinant Fel d 1 has been considered a challenge. The present study shows for the first time that a *Baculovirus* expression system is able to produce an intact rFel d 1 molecule that is glycosylated and structurally equivalent to the natural cat allergen, nFel d 1. Enzymatic digestion of rFel d 1 and further analysis by use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) resulted in a complete coverage of the amino acid sequence of rFel d 1. In addition, the three disulfide bridges at the positions $\alpha 70$ - $\beta 7$, $\alpha 44$ - $\beta 48$, and $\alpha 3$ - $\beta 73$ were verified. The *N*-glycan structure of rFel d 1 was investigated by a combination of MALDI-TOF MS and monosaccharide analysis by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAC). The *N*-glycosylation analyses of rFel d 1 refer to a pattern of glycoforms including core $\alpha 1.3$ -fucosylation that is different from nFel d 1. Further characterization by use of human serum IgE, histamine release, and lymphocyte proliferation assays demonstrated that the immunological characteristics of rFel d 1 are similar to those of nFel d 1. Detailed characterization of both natural and recombinant allergens provides tools to explore immunological mechanisms associated with allergen sensitization and desensitization.

Domestic cat (*Felis domesticus*) is one of the most common household pets, and cat dander is a significant source of indoor allergens. Approximately 20–30% of individuals suffering from allergic asthma respond *in vitro* and/or *in vivo* to cat allergens (1). Sensitization to cat allergens leads into formation of allergen-specific IgE antibodies. Allergic reaction arises when these antibodies are able to facilitate activation of effector cells within the immune system. Activation of effector cells in return leads into release of histamine, eicosanoid synthesis, and cytokine gene expression (2). The subsequent immunological response *per se* is an outcome of cell mediators rather than allergen(s).

Specific allergen immunotherapy has been shown to be ef-

fective in modulating allergic responses and resulting in down-regulation of allergen-specific T-cell responses (3). Conventional specific allergen immunotherapy is performed by the use of standardized natural allergen extracts (4). Cat allergen extract contains a number of allergenic molecules, such as Fel d 1 (5–7), cat albumin (Fel d 2) (8), and cystatin (Fel d 3) (9). Fel d 1, however, has been considered to be the major allergen (1). Immunodominant characteristics have made it a prominent candidate in efforts to develop novel vaccines for treatment of cat allergy (10–14). A foremost ongoing approach is the development of hypoallergenic vaccines, which are based on recombinant technology (3).

Natural Fel d 1 is a noncovalently linked ~38-kDa dimer that is composed of two ~18-kDa subunits. Each subunit comprises α - and β -chains that are encoded by two separate genes (15, 16). Natural Fel d 1 isolated from cat dander is composed of a mixture of variants comprising both full-length and truncated versions of β -chain (17–19). The folding of the polypeptide chains results in an anti-parallel orientation of the α - and β -chain(s) held together with three disulfide bridges (20). Correct orientation of the α - and β -chain(s) is considered to be critical because IgE-related antigenic determinants are conformation-dependent (17–23). Further analyses of the natural molecule have verified that the single *N*-glycosylation site in the β -chain is carrying heterogeneous triantennary complex type of structures. This heterogeneity is caused by terminal sialic acids, fucose, and β -galactose residues (20).

The anti-parallel orientation of the α - and β -chains together with conformation-dependent allergenicity has caused major challenges in efforts to express Fel d 1 as a recombinant (r) allergen. Both the α - and β -chain(s) have been expressed separately in *Escherichia coli* (18, 19, 24, 25). Individual chains, however, show markedly reduced immunogenicity (17–19, 24, 25). Recent studies have focused on expression of Fel d 1 as a fusion protein in a *Baculovirus* expression system (12) and as a His-tagged homodimer in *E. coli* (14). The recently solved three-dimensional structure of a head-to-tail construct expressed in *E. coli* suggests that Fel d 1 belongs to the secretoglobulin family of proteins (26). Prokaryotic expression systems, however, are lacking machinery that are needed for construction of specific post-translational modifications (27). Post-translational modifications such as glycosylation patterns are considered to be important when assessing the structure and immunological properties of recombinant glycoproteins (28).

Here we report the results of the structural characterization of rFel d 1 that was expressed in *Trichoplusia ni* insect cells. In the present study mass spectrometry (MS)¹ and Edman degra-

* This work was part of the Therapeutic recombinant allergens from structural allergology (TRAFSA) project, funded by European Union 5th Framework Programme Grant QLK3-CT-1999-00620. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by a long term postdoctoral fellowship from the Federation of European Biochemical Societies.

|| To whom correspondence should be addressed. Tel.: 45-45-74-81-56; Fax: 45-45-74-86-42; E-mail: MSP@dk.alk-abello.com.

¹ The abbreviations used are: MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; HPAEC-PAC,

TABLE I
Primers used for construction of synthetic Fel d 1 genes

α-Chain									
A1	GAAATCTGCC	CGGCTGTTAA	ACGTGACGTT	GACCTGTTCC	TGACCGGTAC	CCCGGACGAA	TACGTTGA		
A2	CGAGCGTTTT	CCAGAACAAC	CGGCAGAGCT	TTGTACTGAG	CAACCTGTTCC	AACGTATTTCG	TCCGGGGT		
A3	GTTGTTCTGG	AAAACGCTCG	TATCCTGAAA	AACTGCGTTG	ACGCTAAAAT	GACCGAAGAA	GACAAAGA		
A4	TCAGCACAGC	GGGGAGGTGT	AGATTTTGTG	CAGCAGGGAC	AGAGCGTTTT	CTTTGTCTTC	TTCGGTCAT		
ARF	GGTGGTTGCT	CTTCCAACGA	AATCTGCCCG	GCTGTTAA					
ARR	GGTGGTCTGC	AGTCAGCACA	GCGGGGAGGT						
β-Chain									
B1	GTTAAATG	CTGAAACCTG	CCCGATCTTC	TACGACGTTT	TCTTCGCTGT	TGCTAACGGT	AACGAACCTGC	TGCTGGACCT	GTCCC
B2	AACGTAGCAG	TCCTGGATT	TTTTCATAGC	GGTACGTTCC	GGTTCGGTAG	CGTTAACTTT	GGTCAGGGAC	AGGTCCAGCA	GCAGT
B3	AAATCCAGGA	CTGCTACGTT	GAAAACGGTC	TGATCTCCCG	TGTTCTGGAC	GGTCTGGTTA	TGACCACCAT	CTCCTCTCC	AAAGA
B4	TCAACGACCC	AGGGTGTTC	GTTTCAGGTC	TTCAACGGTG	TTCTGAACAG	CTTCACCCAT	GCAGTCTTTG	GAGGAGGAGA	TGGT
BRF	GGTGGTTGCT	CTTCCAACGT	TAAAATGGCT	GAAACCTGCC					
BRR	GGTGGTCTGC	AGTCAACGAC	CCAGGGTGT						

dation was combined to analyze the amino acid sequence and disulfide bridge formation in rFel d 1. The N-glycan structure of rFel d 1 was investigated by combination of MALDI-TOF MS and monosaccharide analysis by HPAEC-PAD. The immunobiological characterization and comparison with nFel d 1 was performed *in vitro* using IgE inhibition, histamine release, and lymphocyte proliferation assays.

EXPERIMENTAL PROCEDURES

Construction of the Synthetic Genes Encoding Fel d 1 Polypeptide Chains—Synthetic genes encoding Fel d 1 α - and β -chains (α : M74952/ β : M77341) were assembled by PCR using overlapping oligonucleotides (Table I). In short, primers A1+A2 and A3+A4 for the α -chain and B1+B2 and B3+B4 for the β -chain were annealed and amplified in six cycles. Extension primers α -chain (ARF/ARR) and β -chain (BRF/BRR) with specific restriction sites were used to rescue the full-length products. The fragments were then cloned into pCR4-TOPO vector (Invitrogen), and their sequences were confirmed (ABI PRISM® 377 DNA sequencer; Applied Biosystems, Framingham, MA).

Construction of Recombinant Baculovirus Vector—The construction of the recombinant Baculovirus vector and the expression of the rFel d 1 was performed by CeNeS Pharmaceuticals plc (Cambridge, UK). The cDNA encoding the mature α - and β -chains were subcloned into Baculovirus shuttle vector pFastBacDUAL (Invitrogen). The mellitin signal sequence was engineered to the N-terminal of both genes by PCR (Fig. 1). The recombinant dual vector was then transformed into MAX efficiency DH10Bac™ competent cells (Invitrogen) containing the baculovirus genome. Within the cell a transposition takes place between a mini-attTn7 target site and the mini-Tn7 element on the vector when recombinant virus is generated. The resulting recombinant bacmid was confirmed according to the suppliers' manual (Invitrogen).

Expression of the rFel d 1—*Spodoptera frugiperda* (Sf21) (Invitrogen) cells were used to generate the primary virus titer for the expression of rFel d 1. The Sf21 cells were grown in 47.5% ExCell 401 (JHR Biosciences), 47.5% TC100 (Invitrogen) and 5% heat-inactivated fetal bovine serum (Invitrogen) as suspension cultures in shaker flask(s) (24). Sf21 cells were transfected with recombinant bacmid DNA in the presence of Lipofectin (Invitrogen) according to the supplier's manual (Invitrogen). The culture medium was collected 7 days post-transfection. The virus titer was assessed by plaque assay (29). For large virus stock production Sf21 cells were infected with the recombinant virus at a multiplicity of infection of 0.5 at a cell density of 1×10^6 cells/ml in a spinner bottle. The virus was harvested 7 days post-infection and titered again by plaque assay. High Five™ cells (Invitrogen) were used for protein production and cultured according to the manufacturer's instruction. For protein production the cells were infected at a multiplicity of infection of 10. The cell supernatant was harvested 96 h post-infection.

Purification of Natural and rFel d 1—Natural Fel d 1 was isolated by use of monoclonal immunoaffinity chromatography from dried cat allergen extract (ALK-Abelló) as described (20). Natural Fel d 1 was then subjected to HR 5/5 Mono Q (Amersham Biosciences) column and eluted with a linear gradient of 0–100% 20 mM Tris-Cl, 0.5 M NaCl, pH 7.5, in 20 min. Eluted fractions containing nFel d 1 were pooled and subjected

to size exclusion chromatography (Amersham Biosciences) in a 10 mM $\text{NH}_4(\text{HCO})_2$ buffer and freeze dried. For the immunological assays, nFel d 1 was dissolved into sterile Dulbecco's phosphate-buffered saline (Invitrogen). Endotoxin levels in the purified Fel d 1 preparations were <15 EU/mg as determined by *Limulus amoebocyte* lysate assay (Bio Whittaker, Walkersville, MD).

Recombinant Fel d 1 was isolated from the High Five™ cell culture supernatant. The cells were gently centrifuged, and the culture supernatant was collected and sterile filtrated. The purification of rFel d 1 followed the protocol described for the nFel d 1.

SDS-PAGE Analysis of Affinity Purified rFel d 1—16% Tris-Tricine SDS-PAGE (Invitrogen) was used to analyze collected fractions (10 μ l/10 ml) from monoclonal affinity chromatography. The electrophoresis was performed in reducing conditions according to the manufacturer's instructions and stained with silver (Invitrogen).

Protein Measurements—A Lambda 800 UV-visible spectrophotometer (PerkinElmer Life Sciences) was used to measure protein concentration at 280 nm using the absorption coefficient A_{280} ($1 \text{ mg} \cdot \text{ml}^{-1} \text{ cm}^{-1}$) = 0.356 for both rFel d 1 and nFel d 1.

Reversed-phase Chromatography—Lyophilized rFel d 1 was first dissolved (47 pmol/ μ l) into Milli-Q grade water (Millipore, Bedford, MA). 5 nmol of rFel d 1 in 0.06% trifluoroacetic acid (Rathburn Chemicals, Peebleshire, UK) was subjected to a 4.6×250 -mm Jupiter, C₈ reversed-phase column (Phenomenex, Torrance, CA). The column was equilibrated with 0.06% trifluoroacetic acid, and the sample was eluted with 0.05% trifluoroacetic acid, 80% acetonitrile (Riedel-de-Hein, Seelze, Germany) in a gradient of 25–55% in 15 column volume and 55–80% in 5 column volume. Recombinant Fel d 1 eluted as a single peak and was collected and dried in a Speedvac. The dried fraction was redissolved into water, divided into 1-nmol aliquots, dried, and stored at -20°C .

The fractionation of the peptide mixtures were performed in a 4.6×250 -mm Jupiter C18 (Phenomenex) column. The peptides were eluted with 0.05% trifluoroacetic acid, 80% acetonitrile in a gradient of 5–45% in 30 column volume or 45–80% in 5 column volume. The separated peptides were dried in a vacuum Speedvac, redissolved into water, and stored at -20°C .

Mass Spectrometric Analyses—Mass spectrometric analysis was performed on a Voyager-DE™ STR Biospectrometry™ (Applied Biosystems, Foster City, CA) MALDI-TOF MS instrument by use of acceleration voltage of 25 kV and a nitrogen laser at 337 nm. The spectra were acquired in the positive ion mode and calibrated externally. MS/MS of selected peptides was performed on a Micromass® Q-Tof Ultima MALDI mass spectrometer (Waters Corporation, Manchester, UK).

20 pmol of intact rFel d 1 was desalted by custom-made micro columns packed with POROS R1/50 (Applied Biosystems, Framington, MA) reversed-phase resin (30). The samples were eluted directly on MALDI targets with 1.0 μ l of matrix solution containing 10 μ g/ μ l α -cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid in 70% acetonitrile, 0.1% trifluoroacetic acid. To analyze the molecular mass of the α - and β -chain, 20 pmol of rFel d 1 was reduced by 45 mM dithiothreitol (Sigma) at 56°C for 30 min and alkylated by 100 mM iodoacetamide (Sigma) for 30 min at room temperature in the dark. Desalting and the MALDI-TOF MS analyses of the sample were then performed as described for the intact protein. Trypsin (NovoNordisk A/S, Bagsvaerd, Denmark), acyl-CoA-binding protein (Sigma), and a Sequazyme™ peptide mass standard kit (Applied Biosystems) were used as external calibrants. Theoretical masses were calculated by GPMW (Lighthouse Data, Odense, Denmark).

Proteolytic Digestions—500 pmol of rFel d 1 was reduced and alkylated as described above. The sample(s) were dissolved into 50 mM

high performance anion exchange chromatography with pulsed amperometric detection; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Pipes, 1,4-piperazinediethanesulfonic acid; PBMC, peripheral blood mononuclear cell.

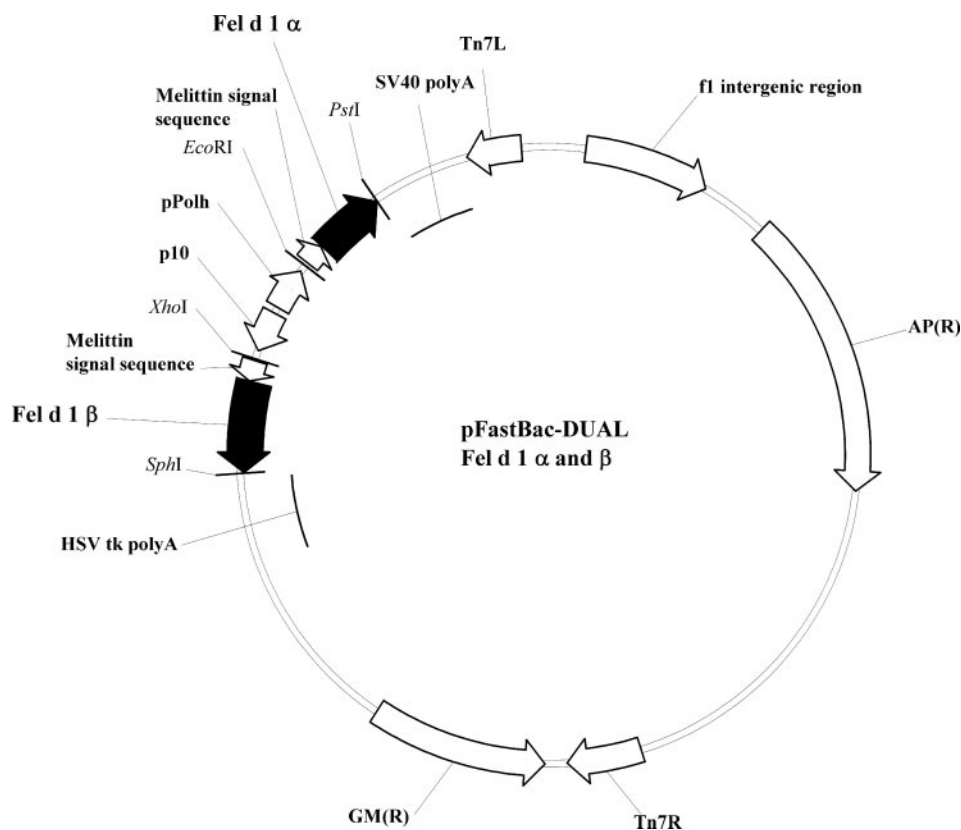


FIG. 1. Construction of the recombinant Baculovirus vector for expression of rFel d 1. Genes encoding α - and β -chain were subcloned into pFastBacDUAL vector under separate (p10/pPolh) promoters. Genes corresponding to Fel d 1 α - and β -chains are colored with black.

Tris-HCl, 0.2 M NaCl, 1 M urea, pH 8.5, and incubated with 3% (w/w) endoproteinase Asp-N (Calbiochem, Sunnyvale, CA) at 37 °C for 18 h (20). Assignment of the disulfide bridges was performed by enzymatic digestion employing endoproteinase Asp-N (Calbiochem) in 50 mM Tris-HCl, 0.2 M NaCl, 1 M urea, pH 8.5, and by sequential digestion with 3% (w/w) trypsin (Sequencing Grade Modified Trypsin, Promega, Madison, WI) and 3% (w/w) chymotrypsin (Wako Chemicals GmbH, Richmond, VA) (20) in 50 mM NH_4Ac , pH 6.0.

Edman Degradation—Sequencing of the rFel d 1-derived peptides was performed with Hewlett Packard G1000A sequencer (Palo Alto, CA) equipped with a Hewlett Packard 1090 series II liquid chromatograph and run as described by the manufacturer.

Acid Hydrolysis and Monosaccharide Analysis—1 mg of rFel d 1 was hydrolyzed in 2 M trifluoroacetic acid at 100 °C for 4 h, subsequently lyophilized in a Speedvac, and resuspended in water. The monosaccharides were analyzed by HPAEC-PAD using a Dionex (Sunnyvale, CA) P-500 chromatographic system consisting of Carbo PacPA-10 preanalytical and analytical columns, an AS50 auto sampler, a GP40 gradient pump, and an ED40 electrochemical detector. The flow rate was 1 ml/min, and the injection volume was 10 μl . Separation was achieved isocratically with water as eluent. A pump with 600 mM NaOH as eluent was used to improve the sensitivity of detection. L-fucose (Merck), D-xylose (Fluka), D-mannose (Merck), D-glucose (Merck), D-galactose (BDH Chemicals Ltd., Poole, UK), GlcNAc (BDH Chemicals Ltd.), N-acetylgalactosamine (BDH Chemicals Ltd.), and D-arabinose (Sigma) were treated as described above and used as standards.

Structural Analysis of the N-Glycan—Removal of N-glycan was assessed with recombinant N-glycosidase F from *Flavobacterium meningosepticum* (Roche Applied Science) and N-glycosidase A from almonds (Roche Applied Science) at 37 °C for 18 h. The resulting products were then analyzed by MALDI-TOF MS (Applied Biosystems) (30). The sequential digestions with glycosidases were performed following the procedure of Kroll-Kristensen *et al.* (20).

IgE Inhibition Analyses—The IgE inhibition experiments were performed on an ADVIA Centaur Immunoassay system (Bayer Diagnostics, Denmark) (31). Pooled human serum IgE from cat-allergic individuals ($n = 4$) was coupled to the solid phase (ADVIA Centaur Universal Reagent Pack, no. 123736), after which the surface was washed with 0.01 M sodium phosphate, 0.1% (w/v) human serum albumin, pH 7.4

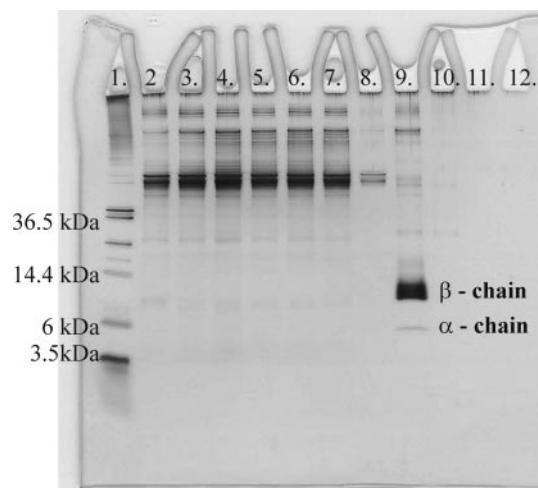


FIG. 2. SDS-PAGE analysis of monoclonal affinity purified rFel d 1 in reducing conditions. Lane 1, molecular weight marker; lanes 2–7, unbound insect cell culture supernatant; lane 8, washed unbound material; lane 9, rFel d 1 α - and β -chain.

(ADIVIA Centaur reagent, no. 1011132). Mixtures of a constant amount of biotinylated cat allergen extract and diluted allergen preparations were added. The amount of biotinylated Fel d allergens bound to the solid phase absorbed IgE was determined as the number of relative light units after the addition of ADVIA Centaur Lite reagent (Universal reagent pack). All of the inhibition experiments were performed as triplicates, and the data sets ($\log_{10}$ (concentration), mean (DoB)) were fitted to a four-parameter logistic function using GraphPad Prism version 4.01 (GraphPad Software, San Diego, CA).

Histamine Release Assay—Histamine release assay was performed using freshly drawn blood from cat-allergic individuals ($n = 4$) and controls ($n = 4$). The tested individuals gave their informed consent to donate blood for research purposes. The antigens, rFel d 1 and nFel d 1,

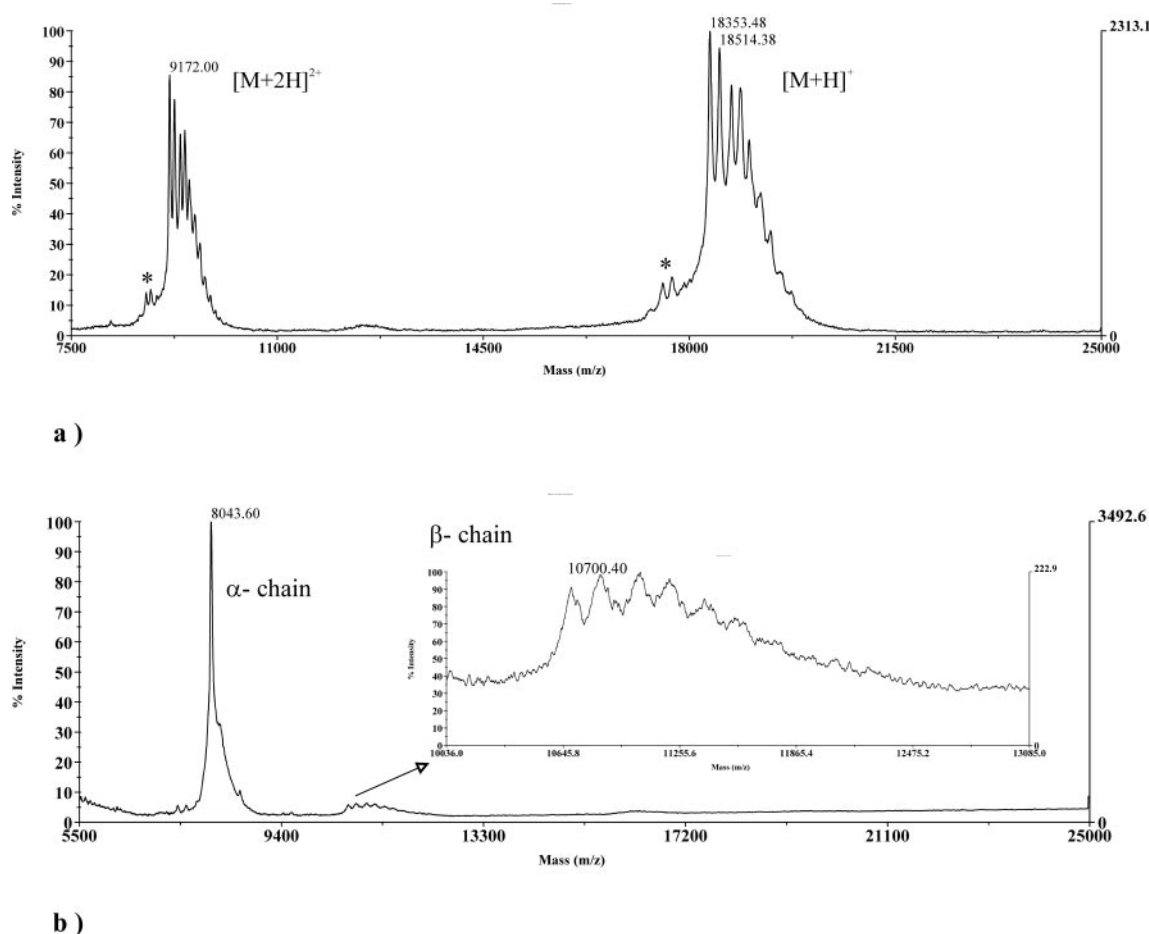


FIG. 3. MALDI-TOF mass spectrometric analyses of rFel d 1. *a*, recombinant Fel d 1 was demonstrated to appear as a heterodimer (upper spectrum). *b*, after the reduction and carboxymethylation, the α - and the β -chains were detected separately (lower spectrum). The m/z values for each peak are included.

were diluted into Pipes buffer (Invitrogen). Antigens were then mixed with the blood samples and incubated for 30 min at 37 °C. Analyses were performed in a final concentration ranging from 1.5 pg/ml to 500 ng/ml of antigen(s). The samples were then centrifuged, and the supernatants were analyzed by enzyme-linked immunosorbent assay (kit IM 2015; Immunotech). The release of histamine was measured at 405 nm by an EL 340 Biokinetics reader (Bio-Tek Instruments, Winooski, VT).

Lymphocyte Proliferation—T-cell lines specific to Fel d 1 were established from peripheral blood mononuclear cells (PBMCs) of cat-allergic patients ($n = 5$) as described previously for grass allergen-specific T-cell lines (32). In short, freshly isolated PBMCs (2×10^6 /ml) were stimulated in 1-ml bulk cultures with natural or rFel d 1 (2 μ g/ml) for 14 days with the addition of recombinant interleukin-2 from day 5. After 14 days T-cells were restimulated with irradiated autologous PBMCs, Fel d 1 (2 μ g/ml), and 0.05 μ g/ml phytohemagglutinin-P (Difco, Detroit, MI), and recombinant interleukin 2 was added at days 3, 4, and 5.

T-cell Stimulation Assay—On day 10 after restimulation, T-cells (2×10^4 /well) were cultured with autologous PBMCs (105/well, irradiated 2500 Rad) in 200 ml of RPMI 1640 medium supplemented with 5% v/v AB serum (Cambrex Bio Sciences), 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Sigma). The culturing was performed with or without antigen in 96-well round-bottomed microtiter plates (Nunc). The cells were cultured for 72 h in a humidified atmosphere at 37 °C and 5% CO₂, followed by a 16-h pulse with 0.5 C [³H]thymidine/well, and thymidine incorporation was determined by scintillation counting. The results are expressed as the mean cpm values of four replicate cultures.

RESULTS

Purification of rFel d 1—Expression of rFel d 1 was demonstrated from the insect cell culture supernatant by SDS-PAGE and Western blotting (data not shown). Monoclonal affinity chromatography was used to capture rFel d 1 from culture supernatant. SDS-PAGE analysis showed that rFel d 1 was

bound to the monoclonal antibody column and eluted in a single step. Both α - and the β -chain were detected when stained with silver (Fig. 2). Affinity purified native rFel d 1 was then analyzed by MALDI-TOF MS, which revealed a major peak in the m/z ranging from 18,000 to 19,000. In addition, a peak at m/z 8000 was seen, indicating overexpression and co-purification of free α -chain (data not shown). Anion exchange and size exclusion chromatography were used to remove the free α -chain and other impurities. For MS analyses, rFel d 1 was subjected to reversed-phase chromatography. The total yield of purified dry weight rFel d 1 was from 1 to 3 mg/liter of culture supernatant. The purity of reduced and nonreduced natural and rFel d 1 was verified by MALDI-TOF MS as described below.

Analysis of Native and Reduced rFel d 1—Analysis of rFel d 1 by MALDI-TOF MS revealed several peaks in the m/z range from 18353.48 to 19377.20 (Fig. 3a). A closer inspection of the mass spectrum revealed spacing between peaks that is diagnostic of glycosylation. In addition, a minor peak (Fig. 3a, asterisk) at m/z ~17,700 referring to nonglycosylated rFel d 1 was detected. Following reduction and *S*-carboxyamidomethylation a peak appeared at 8043.60, and several minor peaks appeared at in the mass range from 10700.40 to 11385.68 (Fig. 3b). The observed signal at 8043.60 was in agreement with the theoretical value m/z 8043.25 for the α -chain. This result indicates to a correct cleavage at the N terminus and overall homogeneous expression of the α -chain.

The peaks in the m/z range 10,700.40 to 11,385.68 showed similar spacing as the native molecule, indicating glycosylation of the β -chain (Fig. 3b). As described below, subsequent diges-

TABLE II
Mass data for peptides derived from endoproteinase Asp-N digest of reduced and carboxymethylated rFel d 1

HPLC fraction	Peptide	Theoretical value	Observed value	Observed difference	Verified by sequencing
F-1	α 1–8	972.53	972.55	+0.02	ND ^a
F-2	α 1–8 ^b	1015.55	1015.50	–0.05	+
F-3	β 72–83	1352.33	1352.6	+0.05	ND
F-4	α 36–45	1216.64	1216.76	+0.12	+
F-5	α 11–25	1724.81	1724.60	+0.21	+
F-6	α 62–70	1096.53	1096.57	+0.04 ^b	+
F-7	β 26–46	2373.28	3248.85		ND
F-8	β 84–92	1029.61	1029.60	–0.01	ND
F-9	β 26–46	2373.28	2373.26	–0.02	ND
F-10	β 60–71	1238.63	1238.68	+0.05	ND
F-11	β 5–11	929.46	929.46	0.00	+
F-12	β 1–11	1358.65	1358.60	+0.05	ND
F-13	α 53–61	1002.55	1002.53	+0.02	ND
F-14	α 46–61	1806.88	1806.89	+0.01	
F-15	α 19–39	2434.30	2434.29	–0.01	ND
F-16	β 47–59	1537.77	1538.73	+0.96 ^b	+
F-17	β 47–59	1537.77	1537.68	–0.09	+
F-18	α 19–35	1964.30	1964.05	–0.25	ND
F-19	α 19–45	3161.67	3161.65	–0.02	ND
F-20	β 12–25	1521.79	1521.60	–0.19	+

^a ND, not done.

^b These mass differences can be explained by the reported modifications.

tion with N-glycosidase A confirmed glycosylation at Asn³³.

Primary Sequence Analysis and Orientation the rFel d 1 α - and β -Chains—Reduced and carboxymethylated rFel d 1 was digested with endoproteinase Asp-N, and the peptides were separated by reversed-phase chromatography. The peptides were identified by N-terminal sequencing, MS-MS, and/or MALDI-TOF MS. These peptides are referred to as F-1 to F-20 in a Table II. Enzymatic digestion with Asp-N gave full sequence coverage for the β -chain. The valine in the α -chain at position 10 was verified from the enzymatic cleavage with combination of trypsin and chymotrypsin (data not shown).

Partial oxidation of the methionine residues was detected in peptides F-7, F-12, and F-13 when analyzed by MALDI-TOF MS, and partial deamidation was found in peptides F-16/F-17. In addition, recombinant Fel d 1 was found to be expressed as a mixture of glycosylated (F-7) and nonglycosylated (F-9) β -chain (Table II). Analyses of the N-glycan will be described below. Modification referring to γ -carboxylation was found from the N terminus of the α -chain (F-2). In N-terminal sequencing, the peptide was found to be blocked, and it was subsequently identified by MS-MS (data not shown). This finding suggests that a subpopulation of rFel d 1 would have modified N termini.

To determine the positions of the disulfide bond linkages, native rFel d 1 was digested with endoproteinase Asp-N and a combination of trypsin and chymotrypsin. Separation of the peptides was performed by reversed-phase chromatography, and all of the resulting peptides were analyzed by MALDI-TOF MS. The combined digestion with trypsin and chymotrypsin revealed disulfide bond linkages between residue(s) α 44– β 48 and α 3– β 73. The third disulfide bond between residue(s) α 70– β 7 was demonstrated by digestion with endoproteinase Asp-N. The observed m/z of this peptide was 2338.25 Da. The disulfide bond formation between all three peptides was verified by reduction with dithiothreitol (Table III). No unpredicted bonds between the cysteine residues and/or free cysteine residues were found.

Analyses of the N-Glycan Structure—Deglycosylation of the rFel d 1 glycopeptide F-7 (Table II) was achieved using N-glycosidase A (Fig. 4). Analysis by MALDI-TOF MS revealed that the expected nonglycosylated peptide was released along with several glycans of different m/z values (Fig. 4a). Deglycosylation of the same glycopeptide (F-7) using N-glycosidase F was not detected. N-Glycosidase A is found active on glycans carrying a

TABLE III
Verification of the disulfide bonds from native rFel d 1

Disulfide bond	Theoretical value	Observed value	Reduced theoretical values	Observed values ^a
α 44– β 48	2156.01	2156.13	648.29 1508.73	ND ^b 1509.68
α 3– β 73	2408.11	2408.58	758.40 1650.72	ND 1651.67
α 3– β 73 ^c	2208.01	2208.13	914.50 1294.52	915.51 ND
α 70– β 7 ^c	2338.12	2338.25	1038.51 1300.62	1039.49 1301.62

^a m/z +1, respectively.

^b ND, not detected.

^c Disulfide bonds found from the endoproteinase Asp-N digest.

α 1.3-linked fucose residue attached to the innermost GlcNAc residue (Fig. 4b). These results suggest that the glycan core is carrying α 1.3-fucosylation, which is in correlation with the previous studies of *T. ni*-expressed recombinant proteins (33).

To further determine the composition of the glycan moiety of rFel d 1, the sample was hydrolyzed, and the released monosaccharides were analyzed by HPAEC-PAD. Fig. 5 shows the HPAEC-PAD chromatogram of monosaccharides released from rFel d 1. The results indicate that the N-glycan is composed of mannose, fucose, GlcNAc, and N-acetylgalactosamine residues. The m/z values and monosaccharide composition of the released glycans was used to predict their structures using GlycoMod (Expasy). The proposed structures are presented in Table IV. Supplementary characterization of the N-glycosidic glycans was performed by MALDI-TOF MS in combination with sequential glycosidase treatment. Removal of the terminal galactose residue was demonstrated by the sequential digestion; however, no evidence of sialic acids was detected (data not shown).

Competition for Allergen-specific IgE Antibodies—The presence of conformational specific IgE epitopes on recombinant Fel d 1 was addressed in an IgE inhibition assay using a serum pool derived from four cat-allergic patients. Serum IgE was captured by anti-IgE immobilized on paramagnetic beads. After washing, the binding of biotinylated cat extract to captured IgE was inhibited by the addition of dilution series of recombinant Fel d 1, natural Fel d 1, and extract, respectively. The inhibition curves determined for cat allergen extract, nFel d 1 and rFel d 1 are shown in Fig. 6. Natural Fel d 1 and rFel d 1

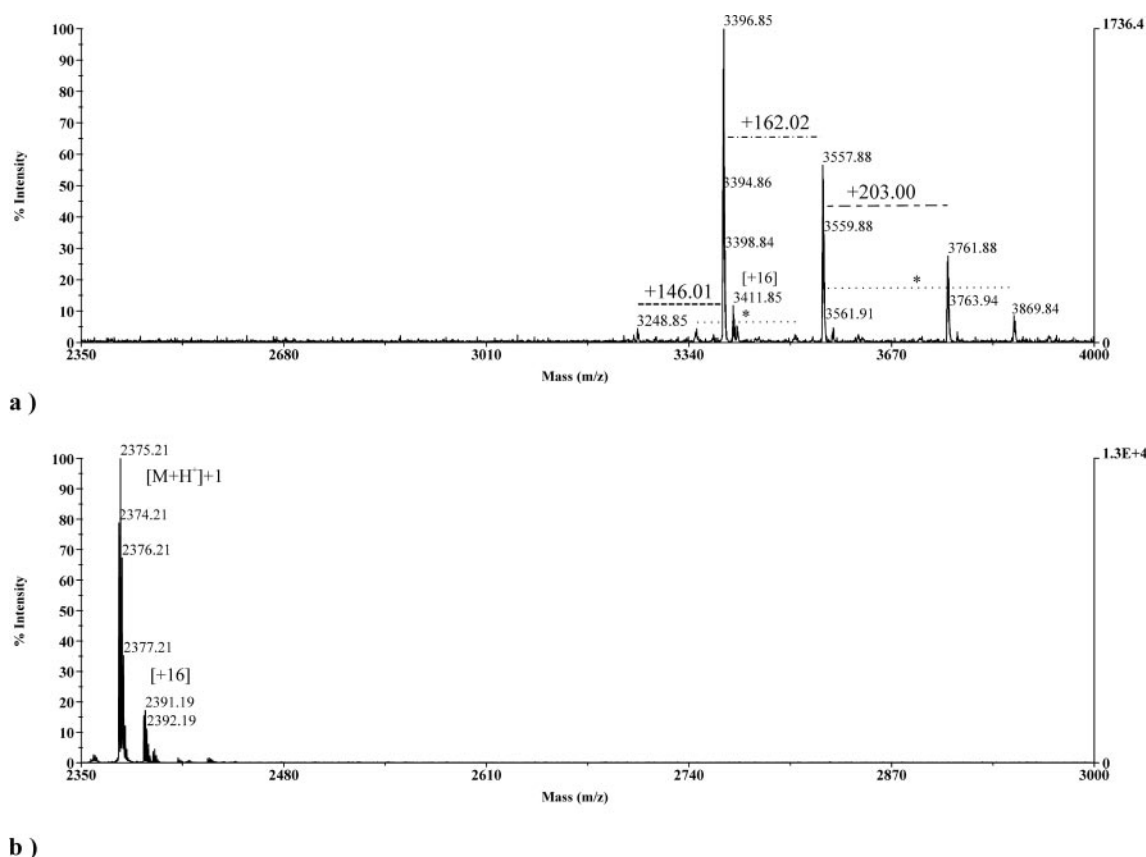


FIG. 4. MALDI-TOF MS analyses of the *N*-glycosidic peptide F-7 from rFel d 1. Spectra of the glycopeptide before (upper spectrum) and after (lower spectrum) enzymatic treatment with *N*-glycosidase A. The masses referring to monosaccharides are included. *, not known.

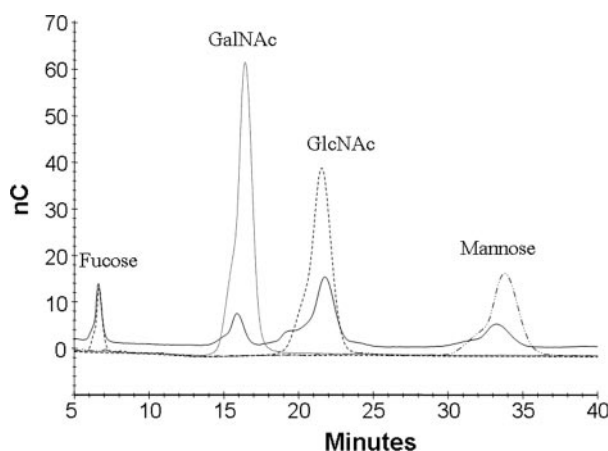


FIG. 5. HPAEC-PAD chromatogram from analysis of monosaccharides released from rFel d 1 after acid hydrolysis. Recombinant Fel d 1 is shown as a solid line. Standards are shown and were used in the following amounts: mannose (dotted and dashed line), 4 μ g; GlcNAc (dotted line), 2 μ g; *N*-acetylgalactosamine (dotted line), 2 μ g; and fucose (dashed line), 2 μ g.

exhibit parallel inhibition curves indicating that the epitope structures of the natural and recombinant allergens are similar. Inhibition by cat allergen extract showed 100% inhibition, and both recombinant and natural Fel d 1 allergens exhibited equal inhibitions (87%) of the interaction between pooled serum IgE and the cat allergen extract. The EC_{50} (inhibitor concentration at 50% inhibition) values determined for the natural and recombinant Fel d 1 preparations differ by $EC_{50}(\text{rFel d 1}) = 1.7 \times EC_{50}(\text{nFel d 1})$, indicating a difference in the concentration of recombinant and nFel d 1 determined at A_{280} .

TABLE IV
Suggested carbohydrate structures from released rFel d 1 glycan
The observed masses all represent $[M + Na]$ or $[M + K]$. NM, no match.

<i>m/z</i> values	Proposed structure
1063.36	[Hex]2[HexNAc]2[Deoxyhexose]2Na
1079.38	[Hex]3[HexNAc]2[Deoxyhexose]1Na
1184.43	[Hex]2[HexNAc]2[Deoxyhexose]2K
1241.45	NM
1387.51	[Hex]4[HexNAc]2[Deoxyhexose]1Na
	[Hex]3[HexNAc]2[Deoxyhexose]2K
	[Hex]4[HexNAc]2[Deoxyhexose]2Na
	[Hex]3[HexNAc]2[Deoxyhexose]3K

Histamine Release Assay—To investigate the biological effect of recombinant and natural Fel d 1 to stimulate basophil, degranulation was tested in histamine release assay using human basophiles derived from individual cat-allergic patients. The release of histamine was found consistent for both recombinant and nFel d 1 in all the four cat-allergic individuals tested, whereas the controls suffering from pollen allergies showed no release. Even though the molar amount of released histamine varied from patient to patient, the histamine response within the patients remained equal between the recombinant and the natural antigen (Fig. 7).

T-cell Proliferation—Allergen-specific T-cell cultures were obtained in most cases when PBMC established from cat-allergic patients were stimulated with natural and recombinant Fel d 1. However, initial stimulation with nFel d 1 resulted in specific T-cell lines in five of five patients, whereas lines were obtained from three of five patients with rFel d 1. When the individual T-cell lines were stimulated with either natural or recombinant Fel d 1, comparable responses were generally obtained even though the response differed between natural and recombinant Fel d 1 in some T-cell lines (Fig. 8). In addi-

tion, all lines examined showed a clear Th2 cytokine profile with a high interleukin 5/interferon- γ ratio (CBA assay; BD Biosciences) (data not shown).

DISCUSSION

The ability to produce recombinant allergens with intact immunochemical properties and correct amino acid sequence

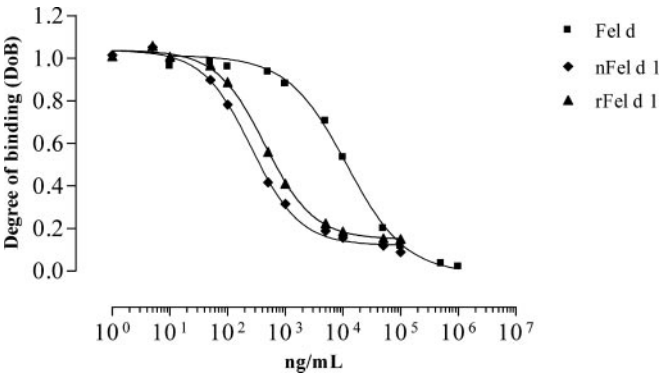


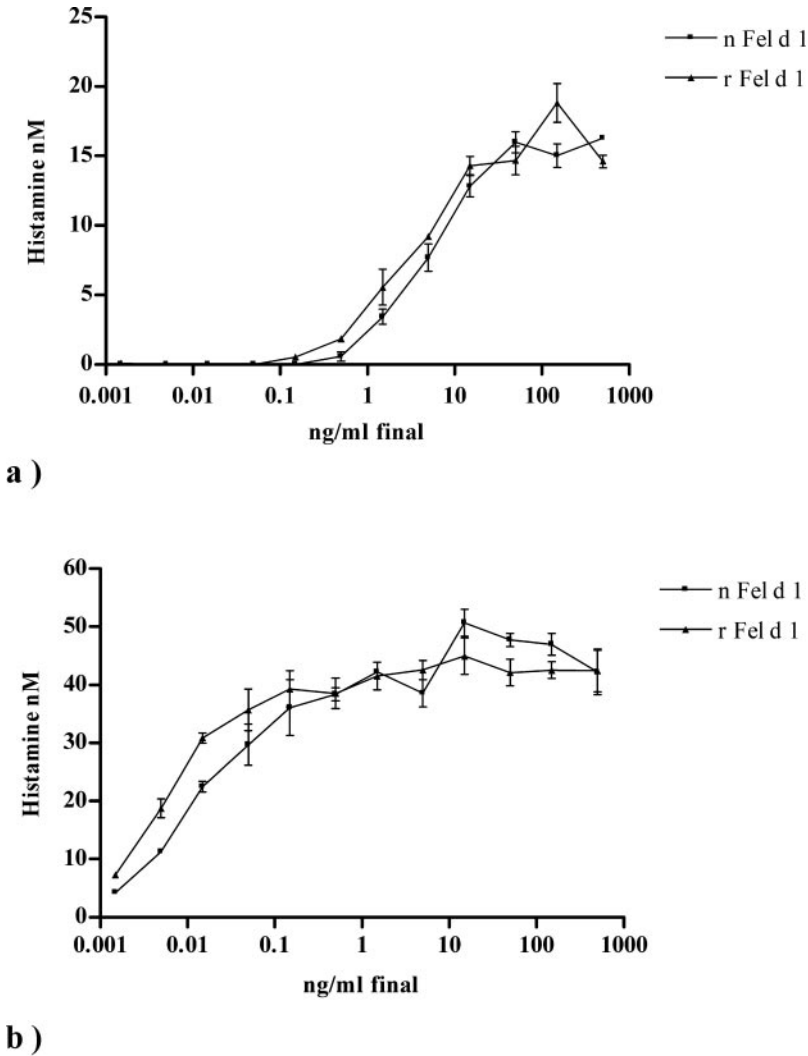
FIG. 6. Inhibition of IgE binding to biotinylated cat allergen extract Fel d by natural Fel d 1, recombinant Fel d 1, and Fel d extract. The individual points are means of triplicate determinations of the degree of binding (DoB) of biotinylated Fel d to IgE, compared with Fel d (■), nFel d 1 (◆), and rFel d 1 (▲). The solid curves represent the four-parameter logistic fits determined for the individual data sets. The curves for nFel d 1 and rFel d 1 are parallel (identical Hill Slope) and nonparallel with the curve fitted for the cat allergen extract data.

and structure is of major importance for their potential use as diagnostic agents and as active ingredients in vaccines. Here we demonstrate the expression of recombinant Fel d 1 as a heterodimer that is structurally and immunochemically equivalent to the naturally occurring cat allergen Fel d 1.

The successful expression of rFel d 1 heterodimer was obtained by cloning the genes encoding Fel d 1 α - and β -chains one by one into a dual gene plasmid (Fig. 1). The expressed α - and β -chains were detected to from the cell culture medium as a mixture of free α -chain and intact α/β -heterodimer. As shown in several previous studies (17–23), monoclonal immunoaffinity chromatography was found to be particularly important in purification of both natural and recombinant Fel d 1. In addition, the co-purification of free α -chain verified that the monoclonal antibody used in the present study was Fel d 1 α -chain-specific. The following purification steps using anion exchange and size exclusion chromatography verified that rFel d 1 shares similar physical and chemical properties with nFel d 1.

The MS analyses of rFel d 1 were designed based on the methods that were previously optimized for nFel d 1 (20). We presumed that expressed as a glycosylated heterodimer, rFel d 1 would show structural characteristics similar to nFel d 1. This was first demonstrated when the three disulfide bridges were assessed. Only after sequential cleavage by trypsin and chymotrypsin were two of the rFel d 1 disulfide bridges (α 44– β 48 and α 3– β 73) verified. Similar results were previously obtained with nFel d 1 (20). The third bridge (α 70– β 7) was demonstrated after cleavage by endoproteinase Asp-N (Table III).

FIG. 7. Specific histamine release in two representative cat-allergic individuals stimulated with rFel d 1 and nFel d 1. The cat allergen-specific IgE of the two individuals were measured by Magic Light (ALK-Abelló). a, 7 SU/ml; b, 34 SU/ml. The release of histamine is comparable with individual total IgE level(s).



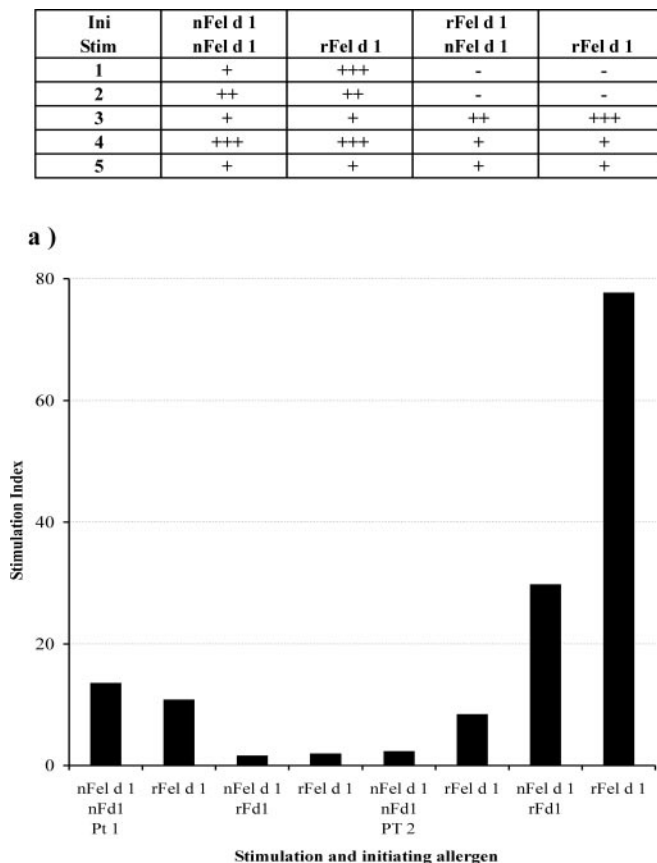


FIG. 8. T-cell lines generated from the peripheral blood of two cat-allergic patients through repetitive stimulation with natural or recombinant Fel d 1. Comparable levels of T-cell activation were found when Fel d 1-specific T-cell lines were initiated with natural and recombinant Fel d 1. The lines were subsequently stimulated with each allergen preparation as indicated for comparison. *a*, T-cell lines from 5 patients scored as follows. *b*, the exact response pattern of two individual patients (SI = cpm in allergen stimulated/cpm in cultures grown in medium alone (background)). +, SI 3; ++, SI > 10; +++, SI > 30.

These results indicate that rFel d 1 is a stable molecule resisting enzymatic cleavage in a manner similar to nFel d 1. Demonstration of the disulfide bridges also showed that rFel d 1 α - and β -chains are in anti-parallel orientation as described for the nFel d 1 (20). Correct orientation of the two-polypeptide chains has been shown to be crucial for the allergenicity of this molecule, because the B-cell epitopes of nFel d 1 have shown to be conformation-dependent (17–19, 21–23). In inhibition assays, natural and rFel d 1 showed similar immunochemical characteristics in competition for cat allergen-specific IgE antibodies. This result suggests that rFel d 1 is able to cover all of the conformational IgE epitopes presented by nFel d 1. This conclusion was further supported by basophil histamine release results. Surface-bound IgE antibodies were stimulated by recombinant and nFel d 1, showing similar dose-dependent release of histamine. Furthermore, the molar release of histamine was found to be consistent when compared with individual total IgE levels (Fig. 7).

Detailed characterization of post-translational modifications from the natural allergen source is necessary before the expression of recombinant allergens. Post-translational modifications can affect the specific activity, recovery, half-life in the circulation, and immunogenicity of proteins (28). Sequence analysis of rFel d 1 by MS showed few but significant modifications within the molecule (Table II, footnote *a*). Partial deamidation

of the asparagine residue was found from peptide F-16 (Table II). Previously deamidation was considered to be a purification artifact. Currently, it is viewed as a signal for protein aging or damage (34). Furthermore, in individuals suffering from celiac disease, deamidation induced by tissue transglutaminase is found to be clinically significant, resulting from immunomodulation of gliadin peptides (35). In the present study, partial deamidation in rFel d 1 may result from extended expression time (96 h). However, considerable changes for the immunogenicity *in vitro* were not detected either in IgE inhibition and/or histamine release assays. Deamidation of rFel d 1 may, however, have influenced the initial stimulation of some T-cell lines, modifying T-cell-specific epitopes (34, 36). On the other hand, comparable results were obtained in many cases when the lines were tested with the two Fel d 1 preparations.

Few groups have explored receptor-specific interactions between allergens and cells that are involved with allergen uptake (37). γ -Carboxylation was first discovered in proteins of the blood coagulation cascade (38), and it has recently been shown to facilitate the interaction of proteins with membrane receptors (39). Because this modification was not previously detected in nFel d 1 (20), the potential immunostimulatory role of γ -carboxylation in addition to deamidation(s) found in the subpopulation of rFel d 1 needs to be further investigated.

Numerous allergens are glycoproteins; however, the role of glycosylation *versus* allergenicity is poorly understood. Furthermore, the studies of glycosylation have mainly been focused on IgE or IgG binding and/or histamine release responses (22–23, 40). In the present study, the rFel d 1 β -chain was expressed with and without *N*-glycosylation (Table II, F-7/F-9). Furthermore, the glycosylation analysis suggests that the *N*-glycosylation profile of the rFel d 1 is different from nFel d 1. Natural cat allergen was shown to carry terminal sialic acids as well as terminal fucose residues, whereas they were not detected when *T. ni* expressed the rFel d 1. In addition, rFel d 1 was shown to carry core α -1.3-fucosylation, which is not found present in nFel d 1 (20). Core α 1.3-fucosylation is commonly found in plant and bee venom allergens (40). Previously studies have suggested that glycosylation is playing important structural and immunobiological roles in nFel d 1 (20, 23). In the present study different glycosylation patterns between natural and rFel d 1 did not show a significant effect for IgE-related immunological assays *in vitro*. However, recent findings by Cobb (41) show that some carbohydrates may facilitate important immunological responses through T-cell activation. Therefore, further studies are needed to elucidate the immunoregulatory role of specific carbohydrate structures in allergy.

Acknowledgments—We thank Gitte Lund from ALK-Abelló, Lene Skov, and Inger Christiansen (University of Southern Denmark) for technical assistance. We thank Dr. Henrik Stålbrand (Department of Biochemistry, Center for Chemistry and Chemical University, Lund University) for access to the HPAEC-PAD system. We also thank Drs. Henrik Jacobi and Kaare Lund from (ALK-Abelló, Research Department) for support for this study.

REFERENCES

- Schou, C. (1996) *Allergy and Allergic Diseases*, 1st Ed., pp. 900–902, Blackwell Sciences, London
- Prussin, C., and Metcalfe, D. D. (2003) *J. Allergy Clin. Immunol.* **111**, S486–S494
- Till, S. J., Francis, J. N., Nouri-Aria, K., and Durham, S. R. (2004) *J. Allergy Clin. Immunol.* **113** 1025–1034
- Lilja, G., Sundin, B., Graff-Lonnevig, V., Hedlin, G., Heilborn, H., Norrind, K., Pegelow, K.-O., and Löwenstein, H. (1989) *J. Allergy Clin. Immunol.* **83**, 37–44
- Ohman, J. L., Lowell, F. C., and Bloch, K. J. (1973) *J. Allergy Clin. Immunol.* **52**, 231–241
- Leitemann, K., and Ohman, J. L. (1984) *J. Allergy Clin. Immunol.* **74**, 147–153
- Löwenstein, H., Lind, P., and Weeke, B. (1985) *Allergy* **40**, 430–441
- Hilger, C., Grigioni, F., and Hentges, F. (1996) *Gene (Amst.)* **169**, 295–296
- Ichikawa, K., Vailes, L. D., Pomes, A., and Chapman, M. D. (2001) *Clin. Exp.*

- Allergy* **31**, 1279–1286
10. Rogers, B. L., Bond, J. F., Craig, S. J., Nault, A. K., Segal, D. B., Morgenstern, J. P., Chen, M.-S., Bizinkauskas, C. B., Counsell, C. M., Lussier, A. M., Luby, T., Kuo, M.-C., Briner, T. J., and Garman, R. D. (1994) *Mol. Immunol.* **13**, 955–966
 11. Maguire, P., Nicodemus, C., Robinson, D., Aaronson, D., and Umetsu, D. T. (1999) *Clin. Immunol.* **93**, 222–231
 12. Vailes, L. D., Sun, A. W., Ichikawa, K., Wu, Z., Sulahian, T. H., Chapman, M. D., and Guyre, P. M. (2002) *J. Allergy. Clin. Immunol.* **110**, 757–762
 13. Oldfield, W. L. G., Larche, M., and Kay, A.B. (2002) *Lancet* **360**, 47–53
 14. Grönlund, H., Bergman, T., Sandström, K., Alvelius, G., Reininger, R., Verdino, P., Hauswirth, A., Liderot, K., Valent, P., Spitzauer, S., Keller, W., Valenta, R., and van Hage-Hamsten, M. (2003) *J. Biol. Chem.* **278**, 40144–40151
 15. Morgenstern, J. P., Griffith, I. J., Brauer, A. W., Rogers, B. L., Bond, J. F., Chapman, M. D., and Kuo, M. (1991) *Proc Natl. Acad. Sci. U. S. A.* **88**, 9690–9694
 16. Griffith, I. J., Craig, S., Pollock, J., Yu, X.-B., Morgenstern, J.P., and Rogers, B. L. (1992) *Gene (Amst.)* **113**, 263–268
 17. Duffort, O. A., Carreira, J., Nitti, G., Polo, F., and Lombardero, M. (1991) *Mol. Immunol.* **28**, 301–309
 18. Bond, J. F., Brauer, A. W., Segal, D. B., Nault, A. K., Rogers, B. L., and Kuo, M.-C. (1993) *Mol. Immunol.* **30**, 1529–1541
 19. Keating, K. M., Segal, D. B., Craig, S. J., Nault, A. K., Semensi, V., Wasserman, A. S., Counsell, C. M., and Bond J. F. (1994) *Mol. Immunol.* **32**, 287–293
 20. Kroll-Kristensen, A. K., Schou, C., and Roepstorff, P. (1997) *Biol. Chem.* **378**, 899–908
 21. Chapman, M. D., Aalberse, R. C., Brown, M. J., and Platts-Mills, T. A. E. (1988) *J. Immunol.* **140**, 812–818
 22. Van't Hof, W., van Milligen, F. J., van den Berg, M., Lombardo, M., Chapman, M.D., and Aalberse, R. C. (1993) *Allergy* **48**, 255–263
 23. Vailes, L. D., Li, Y., Bao, Y., DeGroot, H., Aalberse, R. C., and Chapman, M. D. (1994) *J. Allergy. Clin. Immunol.* **93**, 22–33
 24. Rogers, B. L., Morgenstern, J. P., Garman, R. D., Bond, J. F., and Kuo, M.-C. (1993) *Mol. Immunol.* **30**, 559–568
 25. van Ree, R., van Leeuwen, A., Bulder, I., Bond, J., and Aalberse, R. C. (1999) *J. Clin. Allergy Immunol.* **104**, 1223–1230
 26. Kaiser, L., Grönlund, H., Sandalova, T., Ljunggren, H.-G., van Hage-Hamsten, M., Achour, A., and Schneider, G. (2003) *J. Biol. Chem.* **278**, 37730–37735
 27. Reichert, J. C., and Paquette, C. (2003) *Curr. Opin. Mol. Ther.* **5**, 139–147
 28. Dwek, R. A., Butters, T. D., Platt, F. M., and Zitzmann, N. (2002) *Nat. Rev. Drug Discov.* **1**, 65–75
 29. O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1994) *Baculovirus Expression Vectors: A Laboratory Manual*, pp. 135–136, Oxford University Press, New York
 30. Gobom, J., Nordhoff, E., Mirgorodskaya, E., Ekman, R., and Roepstorff, P. (1999) *J Mass Spectrom.* **34**, 105–116
 31. Petersen, A. B., Gudmann, P., Milvang-Gronager, P., Morkeberg, R., Bogestrand, S., Linneberg, A., and Johansen, N. (2004) *Clin. Biochem.* **37**, 882–892
 32. Wurtzen, P. A., Bufer, A., Wissenbach, M., Madsen, H. O., Ipsen, H., Arved, J., and van Neerven, J. J. (1999) *Clin. Exp. Allergy.* **29**, 1614–1625
 33. Hsu, T.-A., Takahasi, N., Tsukamoto, Y., Kato, K., Shimada, I., Katsuoshi, M., Whiteley, E. M., Fan, J.-Q., Lee, Y. C., and Betenbaugh, M. J. (1997) *J. Biol. Chem.* **272**, 9062–9070
 34. Weintraub, S. J., and Manson, S. R. (2004) *Mech. Ageing Dev.* **125**, 255–257
 35. Arentz-Hansen, H., McAdam, S. N., Molberg, O., Fleckenstein, B., Lundin, K. E., Jorgensen, T. J., Jung, G., Roepstorff, P., and Sollid, L. M. (2002) *Gastroenterology.* **123**, 803–809
 36. Reefer, A. J., Carneiro, R. M., Custis, N. J., Platts-Mills, T. A. E., Sung, S.-S. J., Hammer, J., and Woodfolk, J. A. (2004) *J. Immunol.* **172**, 2763–2772
 37. Chen, C. L., Lee, C. T., Liu, Y. C., Wang, J. Y., Lei, H. Y., and Yu, C. K., (2003) *J. Immunol.* **170**, 528–536
 38. Stenflo, J., Fernlund, P., Egan, W., and Roepstorff, P. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71**, 2730–2733
 39. Walker, C. S., Shetty, R. P., Clark, K., Kazuko, S. G., Letsou, A., Olivera, B. M., and Bandyopadhyay, P. K. (2001) *J. Biol. Chem.* **276**, 7769–7774
 40. Foetisch, K., Westphal, S., Lauer, I., Retzek, M., Altmann, F., Kolarich, D., Scheurer, S., and Vieths, S. (2003) *J. Allergy Clin. Immunol.* **111**, 889–896
 41. Cobb, B. A., Wang, Q., Tzianabos, A. O., and Kasper, D. L. (2004) *Cell* **117**, 677–687