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Cross-reactivity and epitope analysis of Pru a 1, the major cherry allergen

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Abstract

A high percentage of birch pollen allergic patients experiences food hypersensivity after ingestion of fresh fruits and vegetables. The cross-reactivity of the major allergens of sweet cherry (Pru a 1), apple (Mal d 1), pear (Pyr c 1), celery tuber (Api g 1) and carrot (Dau c 1) is due to structural similarities which are reflected by high amino acid sequence identities with Bet v 1a, the major birch pollen allergen. Apart from a strong cross-reactivity to Bet v 1a, IgE inhibition experiments with Mal d 1, Pru a 1 and Api g 1 demonstrated the presence of common and different epitopes among the tested food allergens. Secondary structure prediction of all investigated allergens indicated the presence of almost identical structural elements. In particular, the 'P-loop' region is a common domain of the pollen related food allergens and of pathogenesis related proteins. To identify the IgE binding epitopes, five overlapping recombinant Pru a 1 fragments representing the entire amino acid sequence with lengths of approximately 60–120 residues were investigated. Weak IgE binding capacity was measured exclusively with Pru a 1F4 (1–120) by immunoblotting, whereas none of the fragments showed allergenicity in the rat basophil leukaemia cell mediator release assay. Site-directed mutagenesis experiments with Pru a 1 revealed that amino acid S112 is critical for IgE binding of almost all patients sera tested. This reduced IgE binding was also observed with a single point mutant of Bet v 1a (S112P) and thus indicated serine 112 as an essential residue for preserving the structure of a cross-reactive IgE epitope. Moreover, two Pru a 1 mutants with an altered 'P-loop' region, showed a lowered IgE binding capacity for IgE from a subgroup of allergic patients. The investigation of essential features for preserving cross-reactive IgE-epitopes provides the structural basis for understanding the clinically observed cross-allergenicity between pollen and fruits. Moreover, non-anaphylactic allergen fragments or variants derived from the IgE-inducing pollen allergens may serve as useful tools for a new strategy of specific immunotherapy.

Keywords: Food allergy; Cross-reactivity; Pru a 1; IgE-epitope; Mutant protein

1. Introduction

Birch pollen related food allergies to fresh fruits and vegetables are a known clinical phenomenon in industrialized countries. Approximately 70% of all patients with birch pollinosis exhibit oral symptoms after ingestion of apples, pears, cherries, apricots, hazelnuts or celery tuber, carrots and other vegetables (reviewed by Vieths, 1997). The observed clinical phenomenon is based on respiratory sensitization against birch pollen allergens (Dreborg, 1988; Vieths, 1997). The production of specific IgE from B cells is stimulated by allergen-specific T helper cells type 2 (TH2) and it has been shown that apple extract is a weak stimulator of T cell lines from apple-allergic patients. In contrast, these cells strongly respond to stimulation by birch pollen extract (Fahlbusch et al., 1996). Cross-reaction of the pollen-specific IgE bound to mast cells and basophils with related plant food allergens induces me-

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diator release leading to the type I allergic reaction. The presence of common epitopes on the major allergen of birch pollen (Bet v 1) and related major food allergens, for example Mal d 1 from apple and Pru a 1 from cherry, was clearly demonstrated (Ebner et al., 1991, 1995; Vieths et al., 1994; Scheurer et al., 1997). Moreover, different isoforms of Bet v 1 showed differences in the IgE-binding capaticity. For example Bet v 1a is a strong IgE-binder, whereas Bet v 1 d is a low IgE-binding isoform (Swoboda et al., 1995; Ferreira et al., 1998). Recently, the determination of the three-dimensional structure of Bet v 1 allowed the prediction of three surface areas of the molecule as potential cross-reactive antibody epitopes (Gajhede et al., 1996; Spangfort et al., 1997). To verify the immunodominant IgE epitopes, Bet v 1 was converted into two non-anaphylactic fragments (Vrtala et al., 1997) and modulated by site-directed mutagenesis (Ferreira et al., 1998). The application of hypoallergenic Bet v 1 variants, so-called 'low IgE binders' has been proposed as a possible strategy for T cell specific immunotherapy.

In this study we compared the IgE cross-reactivity of Bet v 1a and the related food allergens from apple, cherry and celery by IgE inhibition experiments with defined recombinant proteins. To identify the relevant IgE epitopes of Pru a 1, recombinant protein fragments were investigated for their IgE binding capacity and their potency to induce mediator release. Site-directed mutagenesis experiments were performed to reveal potential IgE cross-reactive epitopes on Pru a 1 and Bet v 1, and to identify key residues which are essential for the IgE-binding capacity of these allergens.

2. Material and methods

2.1. Patients' sera

All sera were obtained from the sera collection of the Paul-Ehrlich-Institute, Langen, Germany, and the Hospital Borkum Riff, Department of Dermatology and Allergology (Dr H. Aulepp, Borkum, Germany). The sera were taken from patients allergic to birch pollen who reported oral allergy-like symptoms after ingestion of fresh cherries and/or celery. A positive case history of these allergies was confirmed by a positve skin prick test to birch pollen extract, cherry extract and/or celery extract, and by the enzyme allergosorbent test (EAST), with EAST classes ranging from 2–4. Most patients reported oral symptoms upon ingestion of other fruit and vegetables. Three pooled sera (S1: patients allergic to cherry without an allergy to celery, $n=3$; S2: patients allergic to celery without an allergy to cherry, $n=4$; S3: patients allergic to both cherry and celery, $n=3$), with EAST classes ranging from 2–3 for Pru a 1 and Api g 1 were prepared from equal volumes for EAST inhibiton experiments. The pooled patient serum 10.4 was prepared from equal volumes of birch pollen allergic individuals with oral allergy to cherry ($n=4$). For practical reasons, the patients were not identical with the donors of S1, S2 and S3. History of celery allergy was unknown in these patients.

2.2. Allergens

The recombinant major allergens from birch pollen (rBet v 1a), apple (rMal d 1) and celery tuber (rApi g 1) were obtained from BIOMAY, Linz, Austria. Additionally, the recombinant major cherry Histag-fusion protein (fp) and non fusion allergen (rPru a 1), the major apple allergen rMal d 1 (GD) from Golden Delicious, in house rBet v 1 and the major pear allergen (rPyr c 1) (Genbank Acc. No.: AF057030) were purified as described elsewhere (Scheurer et al., 1997). Differing from the referred method rMal d 1 (GD), rBet v 1 and rBet v 1, mutant proteins were expressed using the pET-15b vector system (Novagen, Madison, WI, USA), purified and applied as non fusion proteins after thrombin cleavage.

2.3. IgE immunoblotting and IgE immunoblot inhibition

Allergen extracts, recombinant allergens and *E. coli* lysates were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to (Laemmli, 1970) using the Mini-Protean II cell (BIO-RAD, Munich, Germany). For immunoblot analysis, separated proteins (extract: 18 μg of protein per cm slot; purified allergen: 2 μg per cm slot; $d=1.5$ mm) were electroblotted onto nitrocellulose (NC) membranes (0.45 μm, Schleicher and Schuell, Dassel, Germany) by tank blotting using the BIO-RAD equipment. For immunoblot inhibition experiments 75 μl of the serum pool 10.4 was preincubated with rBet v 1a, rPru a 1, rPru a 1 (fp), rApi g 1 (20 μg of each) and buffer as control for at least 3 h. Thereafter, the sera were diluted to 1 ml and added to blot strips containing the major allergens of birch pollen, cherry or apple, respectively. Immunodetection was performed as described previously (Scheurer et al., 1997; Vieths et al., 1992). The bound enzymatic activity of horseradish peroxidase was detected by enhanced chemiluminescence.

2.4. Enzyme Allergo Sorbent Test (EAST) and EAST inhibition

The determination of allergen-specific IgE was performed in the semi-quantitative EAST using the 'Allergopharma Spez. IgE ELISA' (Allergopharma,

Reinbek, Germany) according to the manufacturers' instructions. The results were expressed as EAST classes ranging from 0 (< 0.35 U/ml IgE = no specific IgE), 1 (0.35–0.7 U/ml IgE); 2 (0.7–3.5 U/ml IgE); 3 (3.5–17.5 U/ml IgE); to 4 (> 17.5 U/ml IgE). The allergens rApi g 1, rBet v 1, rBet v 1 mutants, rPru a 1 and the rPru a 1 mutants were coupled to cyanogen bromide activated filter paper disks (Hycor, Kassel, Germany) at a protein concentration of 1.5 μg/ml (for EAST inhibition) corresponding to a protein amount of 0.25 μg per disk or 0.1 μg/ml (for EAST class determination) corresponding to a protein amount of 1.67 10^{-2} μg per disk as previously described by (Vieths et al., 1995). Seven cherry and birch pollen allergic patients and one non-allergic control were selected for EAST-class determination of the mutant proteins.

For EAST-inhibition, pooled sera S1, S2, and S3 (1:2.5 dilutions) were preincubated with serial dilutions (10^{2}–1.28 10^{-3} μg/ml) of rPru a 1, rMal d 1, rApi g 1 and rBet v 1a for 15 min at room temperature. Thereafter, the EAST was completed according to the standard procedure. Inhibition potencies were calculated as allergen doses responsible for 50% inhibition (ID50) of the EAST signal without inhibitor.

2.5. RBL cell mediator release assay

The β-hexosaminidase release assay is a model for type I allergic reactions which closely corresponds to the results obtained with human basophils. The assay was performed as described in (Hoffmann et al., 1997). In brief, the rat basophil leukaemia cell line, RBL-2H3, passively was sensitized by allergen-specific murine IgE. For sensitization murine sera containing IgE specific for birch pollen extract or Bet v 1 were used. Cross-linking of the $Fc_{\epsilon}RI$-bound IgE with serial dilutions of allergens induces β-hexosaminidase release, which is quantified by an enzymatic colorimetic reaction.

2.6. Secondary structure prediction

The secondary structure predictions were performed with the 'PredictProtein' program (www.embl-heidelberg.de/predictprotein/predictprotein.html) (Rost and Sander, 1994). Protein secondary structure is predicted by a system of neural networks rating at an expected average accuracy > 72% for the three states helix (H), strand (E) and loop (L).

2.7. Circular dichroism and homology modeling

Circular dichroism (CD)-spectra of Pru a 1 after CNBr cleavage (Boehm and Rösch, 1997) of the fusion protein were recorded on a Jasco J-600 spectropolarimeter with a stepwith of 0.2 nm and a bandwith of 1 nm. The spectral range was 185–255 nm. Three scans were accumulated at a temperature of 22°C. The concentration of Pru a 1 was 15 μM in 10 mM KH_2PO_4, pH 7.

The structure of Pru a 1 was obtained by homology modeling using the program SWISS-MODEL (http://www.expasy.ch/swissmod/SWISS-MODEL.html) with the X-ray structure of Bet v 1 (pdb accession code 1BV1) as a template, followed by 100 steps of a Powell energy minimization using the standard Tripos force field provided by SYBYL 6.4 software (Tripos, Inc.). For visualization of the tertiary structure the program MolScript v2.1.2 was used.

2.8. Cloning of Pru a 1 fragments

The cDNAs coding for the Pru a 1 fragments were generated by PCR amplification of Pru a 1 cDNA as template using following oligonucleotide primers (Eurogentec, Seraing, Belgium): Pru a 1F1(1-69): For: 5′-GGT GTC TTC ACA TAT GAG-3′, Back: 5′-TAT TAC TTG TGC TTC ACG TAG CC-3′; Pru a 1F2(43-120): For: 5′-GAA ATC CTT GAA GGA GAT GGC-3′, Back: 5′-TAT TAG TGG TAG TGG CTG GTG C-3′; Pru a 1F3(100-159): For: 5′-GAG ACC AAG TTG GTG GC-3′, Back: 5′-CTC GAG TTA GTT GTA GGC ATC GGG GTG GCC-3′, Pru a 1F4(1-120): For: 5′-GGT GTC TTC ACA TAT GAG-3′, Back: 5′-TAT TAG TGG TAG TGG CTG GTG C-3′; Pru a 1F5(43-159): For: 5′-GAA ATC CTT GAA GGA GAT GGC-3′, Back: 5′-CTC GAG TTA GTT GTA GGC ATC GGG GTG GCC-3′. Stop codons are underlined. After a hot start, the PCR (35 cycles) was performed for 1 min at 94°C, 1 min at 52°C, 1 min at 72°C and a final extension for 10 min at 65°C. The purified PCR-fragments were ligated into pSCREEN-T (Novagen, Madison, WI, USA) coding for a large fusion polypeptide of 39 kDa which is thought to facilitate improved protein expression of small peptides. *E. coli* strain NovaBlue (DE3) (Novagen, Madison, WI, USA) was transformed with plasmids by heat shock according to the manufacturers' instructions. Positive clones were selected by PCR screening with the oligonuleotide primers T7-gen: 5′-TGA GGT TGT AGA AGT TCC G-3′ or T7-terminator: 5′-GCT AGT TAT TGC TCA GCG G-3′ and one of the gene-specific primers described above.

2.9. Site-directed mutagenesis of Pru a 1

Single amino acid substitutions (T10P, G46P, S112P, S112C), amino acid deletion (ΔT52) and double amino acid substitution (SI112/113CV) were performed by site-directed mutagenesis of Pru a 1. Genetic engineering of Pru a 1 (T10P) was performed with the oligo-

nucleotide primers PRU5XHO(T10P): 5′-ACT TCG A*CT CGA G*GG TGT CTT CAC ATA TGA GAG CGA GTT CCC CTC TGA G-3′ and PRU3XHO+: 5′-ACT TCG A*CT CGA G*TT AGT TGT AGG CAT CGG GGT GGC C-3′ using Pru a 1 cDNA as template. Stop codons are underlined, the Xho I cleavage site is printed in italics. After a hot start, the PCR (35 cycles) was performed for 1 min at 94°C, 1 min at 60°C, 1 min at 72°C and a final extension for 5 min at 65°C. After Xho I cleavage the purified cDNA was cloned into plasmid pET-16b (Novagen, Madison, WI, USA). Initial cloning was done in *E. coli* XL1-Blue (Stratagene, Heidelberg, Germany). Positive clones were selected by PCR screening with the oligonucleotide primers T7-promoter: 5′-TAA TAC GAC TCA CTA TAG G-3′ and PRU3XHO: 5′-*CTC GAG* TTA GTT GTA GGC ATC GGG GTG GCC-3′.

Further site-directed mutagenesis experiments were performed with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany) according to the manufacturers instructions using the construct pET-16b/Prua1 as template. The following mutagenesis primers were used: G46P-For: 5′-CTG AAA TCC TTG AAC CAG ATG GCG GCC CCG-3′ and G46P-Back: 5′-CGG GGC CGC CAT CTG GTT CAA GGA TTT CAG-3′; ΔT52-For: 5′-GAT GGC GGC CCC GGA (ACC) ATC AAG AAG ATC AC-3′ and ΔT52-Back: 5′-GTG ATC TTC TTG AT(GGT)A CCG GGG CCG CCA TC-3′; S112P-For: 5′-CCA GCG GAG GAC CCA TCA TCA AGA GC-3′ and S112P-Back: 5′-GCT CTT GAT GAT GGG TCC TCC GCT GG-3′; S112C-For: 5′-CCA GCG GAG GAT GCA TCA TCA AGA GC-3′ and S112C-Back: 5′-GCT CTT GAT GAT GCA TCC TCC GCT GG-3′; I113 V-For: 5′-CCA GCG GAG GAT CCG TCA TCA AGA GC-3′ and I113 V-Back: 5′-GCT CTT GAT GAC GGA TCC TCC GCT GG-3′; SI112/113CV-For: 5′-CCA GCG GAG GAT GCG TCA TCA AGA GC-3′ and SI112/113CV-Back: 5′-GCT CTT GAT GAC GCA TCC TCC GCT GG-3′. Basepair substitutions (and deletions) are underlined. After a hot start, the PCR (16 cycles) was performed for 30 s at 95°C, 1 min at 55°C, 12 min 22 s at 68°C and 10 min at 68°C as final extension. All oligonucleotide primers were HPLC purified and purchased from ARK, Darmstadt, Germany.

2.10. Site-directed mutagenesis of Bet v 1

Site-directed mutagenesis of Bet v 1 was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Heidelberg, Germany). The oligonucleotide primers BET-P151L-Back: 5′-TTA GTT GTA GGC ATA GGA GTG TGC CAA GAG GTA GCT CTC AAC GGC-3′ and BET-P151L-For: 5′-GCC GTT GAG AGC TAC CTC TTG GCA CAC TCC GAT GCC TAC AAC TAA-3′ were applied for amplification of Bet v 1 (S112P) using the clone Bet v 1 (S112P,L151P) as template. PCR was performed as described above. All oligonucleotide primers were HPLC purified and purchased from ARK, Darmstadt, Germany. The Bet v 1 (S112C) variant was amplified by a triple PCR strategy using the clone Bet v 1 (S112P,L151P) as template. The following primers were used: BET5NDE: 5′-GGA ATT CCA TAT GGG TGT TTT CAA TTA CGA AAC TGA G-3′ and BET-P112C-Back: 5′-CTT GTT GCT GAT CTT CAA GAT GCA TCC TCC ATC AGG GGA-3′; BET-P112C-For: 5′-ACC CCT GAT GGA GGA TGC ATC TTG AAG ATC AGC AAC AAG-3′ and BET3XHO/P151L: 5′-CCG CTC GAG TTA GTT GTA GGC ATC GGA GTG TGC CAA GAG GTA-3′. Basepair substitutions are underlined. An initial PCR (30 cycles) was performed for 1 min at 95°C, 1 min at 50°C, 1 min 72 s at 68°C and 5 min at 65°C as final extension. After a hot start, an additional assembly PCR of the two purified overlapping cDNAs was performed at 1 min at 94°, 1 min at 50°C, 1 min at 72°C. After eight cycles terminal primers were added and additional PCR (30 cycles) was performed at 1 min at 94°C, 1 min at 50°C, 1 min at 72°C and 65°C as final extension.

2.11. DNA sequencing

DNA sequence analyses were performed with an ABI 373 'stretch' automatic fluorescent sequencer (Applied Biosystems, Weiterstadt, Germany) using the vector specific primers T7-promoter and T7-terminator or the gene specific primers PRU3XHO and PRU5XHO: 5′-ACT TCG A*CT CGA G*GG TGT CTT CAC ATA TGA GAG CGA G-3′.

2.12. Protein expression and purification

Protein expression in *E. coli* NovaBlue or BL21(DE3) was induced by adding isopropyl-D-thiogalactopyranoside (IPTG) to LB-medium to a final concentration of 1 mM. The recombinant fusion proteins were purified by nickel-nitrilotriacetic (Ni-NTA) metal-affinity chromatography as described recently (Scheurer et al., 1997). Enzymatic- or CNBr-cleavage (Boehm and Rösch, 1997) of the purified proteins were performed to obtain non-fusion proteins.

3. Results

3.1. Cross-reactivity

IgE immunoblot inhibition experiments were performed with the serum pool 10.4 from four birch pol-

Table 1
IgE immunoblot inhibition. Total protein of allergen extracts and recombinant allergen were separated by SDS-PAGE and blotted onto NC-membranes. Preincubation of pooled patient serum 10.4 was performed with rBet v 1a, rPru a 1 (fp), rPru a 1 and rApi g 1. Decreased IgE binding was estimated from the band intensities in relation to the non-inhibited control (nd, not determined)

Solid phase	Inhibitor			
	rBet v 1	rPru a 1 (fp)	rPru a 1	rApi g 1
rPru a 1 (fp)	100%	100%	100%	0%
Cherry extract	100%	100%	nd.	0%
Apple extract	100%	90%	90%	0%
rBet v 1	100%	0%	nd.	0%
Birch pollen extract	100%	0%	nd.	0%

len allergic patients suffering from oral allergy syndrome against cherry. Electrophoretically separated rPru a 1 (fp), cherry extract, apple extract, rBet v 1 and birch pollen extract were blotted on NC and used as solid phase. The serum pool was preincubated with rBet v 1, rPru a 1 (fp), rApi g 1 or buffer as control, respectively. For blotted rPru a 1 (fp) and apple extract, an additional IgE inhibition test was performed with the rPru a 1 non-fusion protein. Preincubation with rBet v 1 prevented the binding of IgE to all allergens tested, whereas no reduction of the IgE binding was observed with rApi g 1. The preincubation with rPru a 1 (fp) showed a complete self-inhibition of IgE binding, a reduction of the IgE binding of approximately 90% to the major apple allergen and no inhibiton of the IgE binding to rBet v 1. The rPru a 1 non-fusion protein and the rPru a 1 fusion protein showed the same IgE binding reactivity (Table 1). All inhibiton values were estimated from the band intensities in relation to the non-inhibited control.

For EAST inhibition experiments, three pooled sera were tested with rPru a 1 and rApi g 1 bound to filter paper disks. All three sera had a positive EAST of class 2 or 3 to both the celery and the cherry allergen. The inhibition of the IgE binding to rPru a 1 was investigated with pooled sera S1 (patients allergic to cherry without an allergy to celery) and S3 (patients allergic to both cherry and celery), respectively. The inhibition of the IgE binding to rApi g 1 was determined with pooled sera S2 (patients allergic to celery without an allergy to cherry) and S3, respectively. Preincubation of the pooled sera was performed with serial dilutions of rPru a 1, rMal d 1, rApi g 1 and rBet v 1a, respectively, at protein concentrations ranging from $1.28\ 10^{-3}$–10^{2} μg/ml (Fig. 1).

IgE binding to Pru a 1 was almost completely inhibited after preincubation of S1 and S3 with rPru a 1 (10^{2} μg/ml) with 50% inhibition doses (ID50) calculated between 1 and 10 μg/ml (Fig. 1B). In contrast, preincubation with a low concentration of rBet v 1 ($3.2\ 10^{-2}$ μg/ml) still resulted in an inhibition of 75%. The ID50 value was calculated by the maximal Bet v 1 dilution ($1.28\ 10^{-3}$ μg/ml) (Fig. 1A). Only approximately 50% IgE inhibition to rPru a 1 was measured after preincubation of both pooled sera with rMal d 1 ($ID50 = 10^{2}$ μg/ml) (Fig. 1A), whereas no significant inhibition of IgE binding was detected after preincubation with rApi g 1 at the highest concentration (Fig. 1B). Preincubation of pooled sera S2 and S3 with rPru a 1 and rMal d 1 induced no reduction of IgE binding to Api g 1 (Fig. 1C and D). In contrast, a nearly complete inhibition of antibody binding to rApi g 1 resulted after preincubation with rApi g 1 (10^{2} μg/ml) (Fig. 1D) and rBet v 1a even at a very low allergen concentration of $6.4\ 10^{3}$ μg/ml (Fig. 1C). For S2 and S3 preincubated with rApi g 1, 50% IgE inhibition was calculated at an allergen concentration of 4 μg/ml (Fig. 1D), whereas inhibition by rBet v 1a reached 50% at maximal allergen dilution (Fig. 1C).

To investigate the cross-reactivity between the major food allergens and Bet v 1 in a biological model of the type I reaction, the murine in vitro mediator release assay was applied. Rat basophil leukaemia cells (RBL-2H3) were passively sensitized with an IgE-containing serum (pool 5) raised by immunisation of mice with birch pollen extract. Mediator release was induced after cross-linking of receptor bound IgE with serial dilutions of rBet v 1, rApi g 1, rMal d 1 (GD), rPyr c 1 and rPru a 1 (Fig. 2). After cross-linking with rBet v 1 at a protein concentration of 10^{-2} μg/ml, a maximal release of 50% was observed. Both Bet v 1 molecules, Bet v 1a derived from BIOMAY and the in house Bet v 1 showed an identical reactivity. Moreover, a maximal mediator release in the same order of magnitude, except for rPyr c 1 (approximately 31%), was measured for rApi g 1 (49%), rPru a 1 (52%) and rMal d 1 (GD) (41%) at a protein concentration of 10 μg/ml (rPru a 1, rApi g 1, rMal d 1 (GD)). The slopes of all curves were similar.

3.2. Amino acid sequence comparison and secondary structure prediction

All investigated food allergens show high sequence identities with Bet v 1a (Acc. No.: X15877), ranging from 55–59% for Pru a 1 (Acc. No.: U66076) from Prunoideae, and Mal d 1 (Acc. No.: Z48969) and Pyr c 1 (Acc. No.: AF057030) from Maloideae, and approximately 40% for Dau c 1 (Acc. No.: Z84376) and Api g 1 (Acc. No.: Z48967) from Apiaceae. The amino acids 46–54 of Bet v 1a representing the glycine-rich P-loop region followed by a conserved lysine residue (GXGGXGXXK) (Spangfort et al., 1997) are also highly conserved in all analysed birch pollen related food allergens (Fig. 3). Secondary structure predictions of Bet v 1a and the birch pollen related food allergens

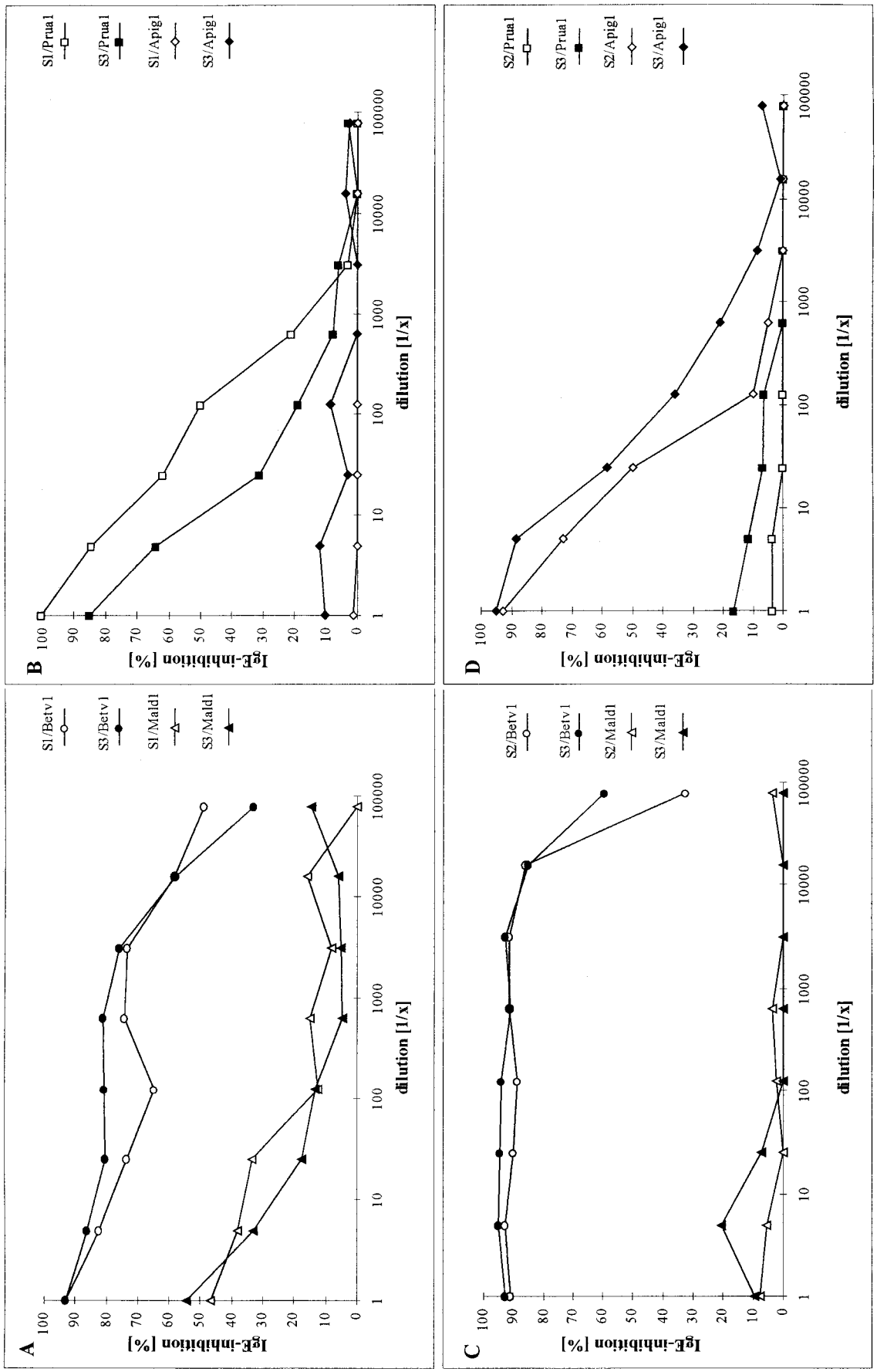

Fig. 1. EAST inhibition experiments. Inhibition of IgE binding from pooled patient sera S1 and S3 to immobilized Pru a 1 (A and B) and from pooled patient sera S2 and S3 to immobilised Api g 1 (C and D). Preincubation of sera with rBet v 1a (—○—, —●—), rMal d 1 (—△—, —▲—), rPru a 1 (fp) (—□—, —■—) and rApi g 1 (—◇—, —◆—). Dilution 1 corresponds to an allergen concentration of 100 μg/ml. The inhibition was calculated in relation to the uninhibited control.

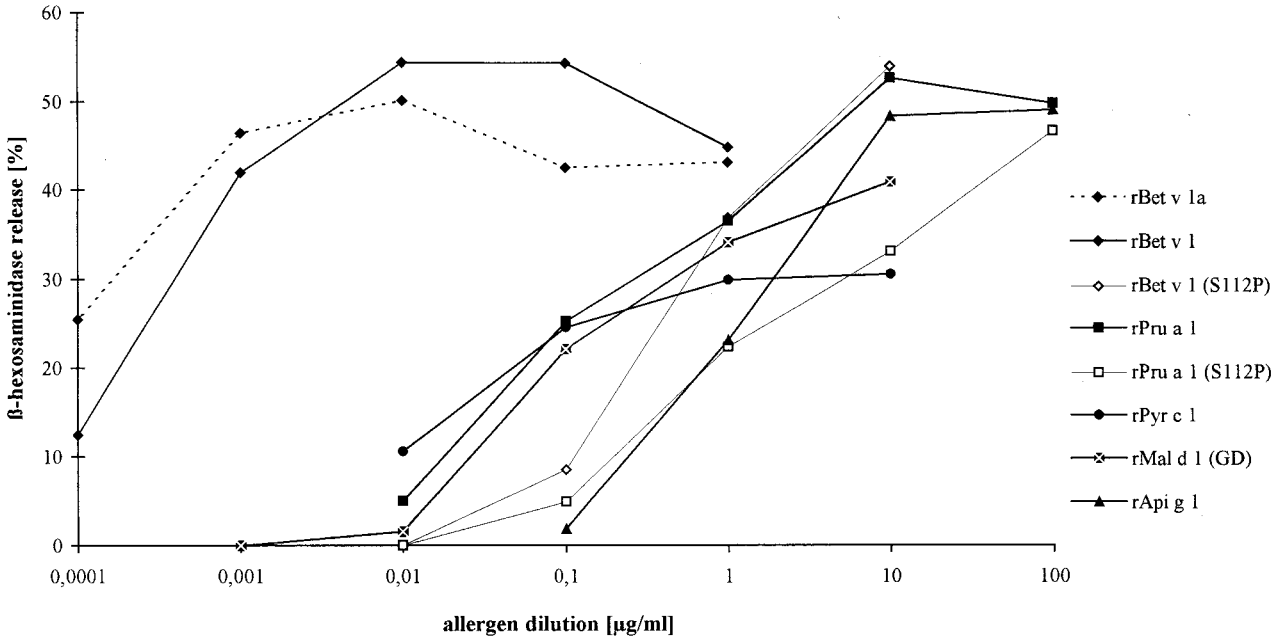

Fig. 2. β-hexosaminidase release assay. RBL-2H3 cells were passively sensitised with murine IgE raised by immunisation with birch pollen extract. Cross-linking of receptor-bound antibodies was performed with rBet v 1 (rBet v 1a and in house rBet v 1), rPru a 1, rApi g 1, rPyr c 1 and rMal d 1 (GD). Moreover, the mutants rPru a 1 (S112P) and rBet v 1 (S112P) were investigated. All allergens were applied as non-fusion proteins.

Pru a 1, Mal d 1, Pyr c 1, Dau c 1 and Api g 1 were compared using the program 'PredictProtein'. All analysed allergens revealed an almost identical secondary structural pattern (Fig. 3). It comprises six β-sheets and three helices, except for Dau c 1 and Api g 1 in amino acid positions 68–73 and for Mal d 1 and Pru a 1 in the amino acid residues 39–45, where no structure segment was predicted. When compared to the known structure of Bet v 1 (Gajhede et al., 1996) an incorrect prediction was obtained from the Bet v 1 sequence for the α-helix 2 (25–34) and β-strand 4 (66–75). Moreover, no helical structure could be observed in the amino acid position 93–97 of the X-ray structure of Bet v 1.

3.3. CD-spectra and homology modeling of the Pru a 1 structure

CD-spectra of rPru a 1 and rBet v 1 were recorded at 22°C and appeared to be largely superimposed (Fig. 4A and B). This, together with the prediction of similar positions of secondary structure elements and the high degree of sequence identity (59.1%), indicates that the two proteins are structurally related. We, therefore, generated the structure of Pru a 1 by a homology modeling approach using the X-ray structure of Bet v 1 (pdb code 1BV1) as a template. The obtained 3D-structure of Pru a 1 (Fig. 5) was similar to Bet v 1 (Gajhede et al., 1996) with respect to the arrangement of the seven-stranded β-sheet, as well as the two N-terminal and the long C-terminal α-helix.

3.4. Epitope analysis

To localize the IgE-binding epitopes equal amounts of induced rPru a 1 fragments and rPru a 1 fusion protein were investigated by IgE immunoblotting after electrophoretic separation of *E. coli* lysate with the pooled patient serum 10.4 (Fig. 6A) and the serum from a non-allergic control (Fig. 6B). Weak IgE binding was observed with the fragments F1, F2, F4 and F5, with apparent molecular masses between 47 and 52 kDa, in comparison to the 55 kDa full-length protein, generated as large fusion proteins by application of the pSCREEN-T expression system. No IgE binding to the target protein was detected with the control serum, whereas non-specific binding of the antibody was observed to bacterial proteins in the positive and negative controls. In contrast to the full length Pru a 1 fusion protein, all allergen fragments induced a negligible mediator release in the RBL-cell assay passively sensitized with anti-birch pollen IgE (results not shown).

Seven Pru a 1 mutants and two Bet v 1 mutants were generated by site-directed mutagenesis. The immunoreactivity of these mutants was compared to the corresponding wild type proteins by IgE immunoblotting experiments under reducing conditions. The mutant proteins containing single amino acid substi-

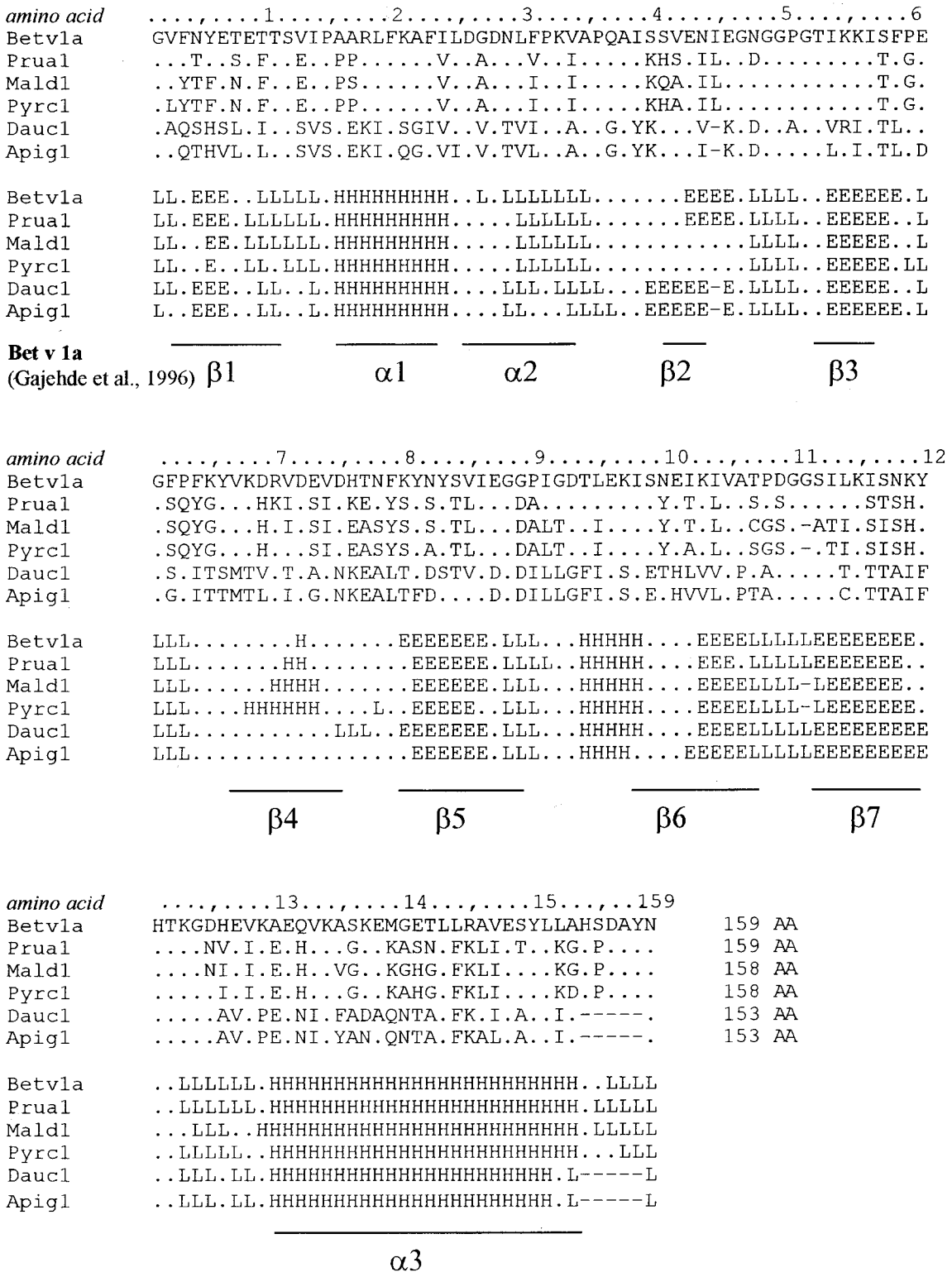

Fig. 3. Secondary structure prediction (L: loop; H: helix; E: sheet) of the birch pollen associated food allergens Pru a 1, Mal d 1, Pyr c 1, Dau c 1, Api g 1 and Bet v 1a, the major birch pollen allergen using "PredictProtein". Blanks in the amino acid sequence indicate identical residues, blanks in the predicted structure indicate an average accuracy $<72\%$. The structural elements of Bet v 1a determined by X-ray spectroscopy (Gajhede et al., 1996) are indicated.

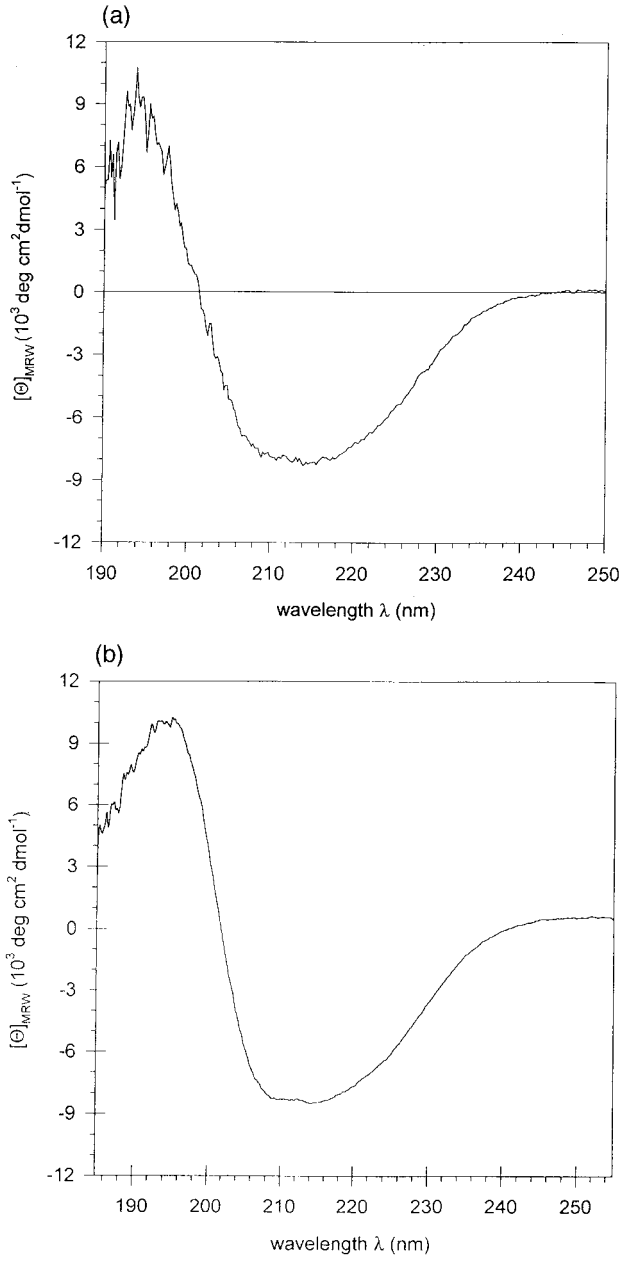

Fig. 4. CD-spectrum of 15 μM Pru a 1 (A) and reference spectrum of Bet v 1 (B) at 22°C recorded on a Jasco J-600 spectropolarimeter (stepwidth 0.2 nm; bandwidth 1 nm).

tutions, one double amino acid substitution or one amino acid deletion were generated to compare the IgE binding activity with the wild-type cherry allergen (Fig. 7A). Amino acids for mutagenesis were selected by comparing the sequences of Pru a 1 with Bet v 1a and the low IgE binding isoform Bet v 1d. Those residues (Ser112 and Ile113) identical in both strong IgE binding proteins Pru a 1 and Bet v 1a and differing in the low IgE binding isoform Bet v 1d were substituted in the cherry protein. Moreover, Gly46 and Thr52 were selected for mutagenesis of the P-loop region.

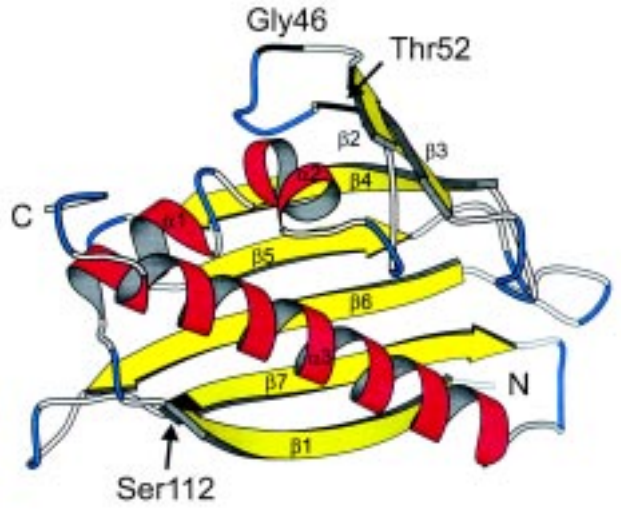

Fig. 5. Model of the three-dimensional structure of Pru a 1 prepared by the program SWISS-MODEL using the known X-ray structure of Bet v 1 as template. The sevenstranded β-sheet and three α-helices are indicated. Additionally, the amino acids Gly46 and Thr52 which belong to the exposed P-loop region and Ser112 which is the first amino acid of the β7-strand, are labeled.

The conserved residue Thr10 has recently been proposed as putative important residue for the IgE binding of the major allergen of hazel, Cor a 1 (Ferreira et al., 1996). Considering the high degree of identity (58%) between the cherry protein and Cor a 1, the rel-

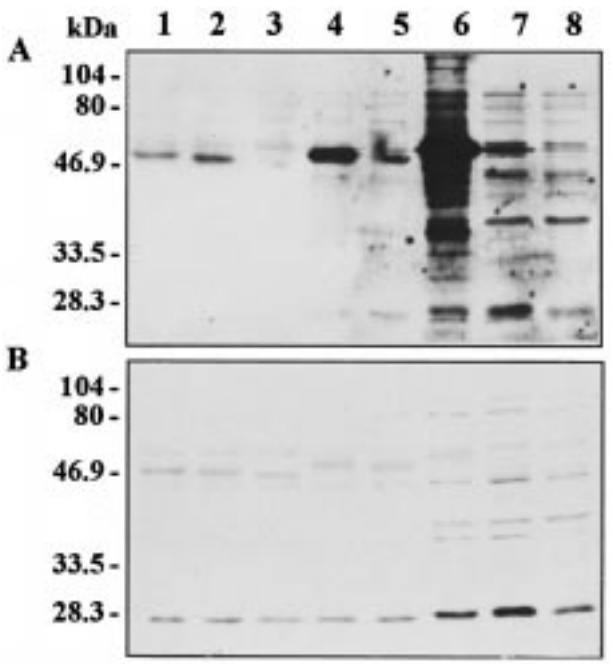

Fig. 6. IgE immunoblot of rPru a 1 fusion fragments (1: F1(1-69); 2: F2(43-120); 3: F3(100-159); 4: F4(1-120); 5: F5(43-159)), rPru a 1 fusion protein (6), plasmid control (7) and bacterial lysate (8) with the patient pool serum 10.4 (A) and the negative control serum PEI 22 (B). All fragments were cloned in the pSCREEN-T vector system which encodes a fusion polypeptide of 39 kDa.

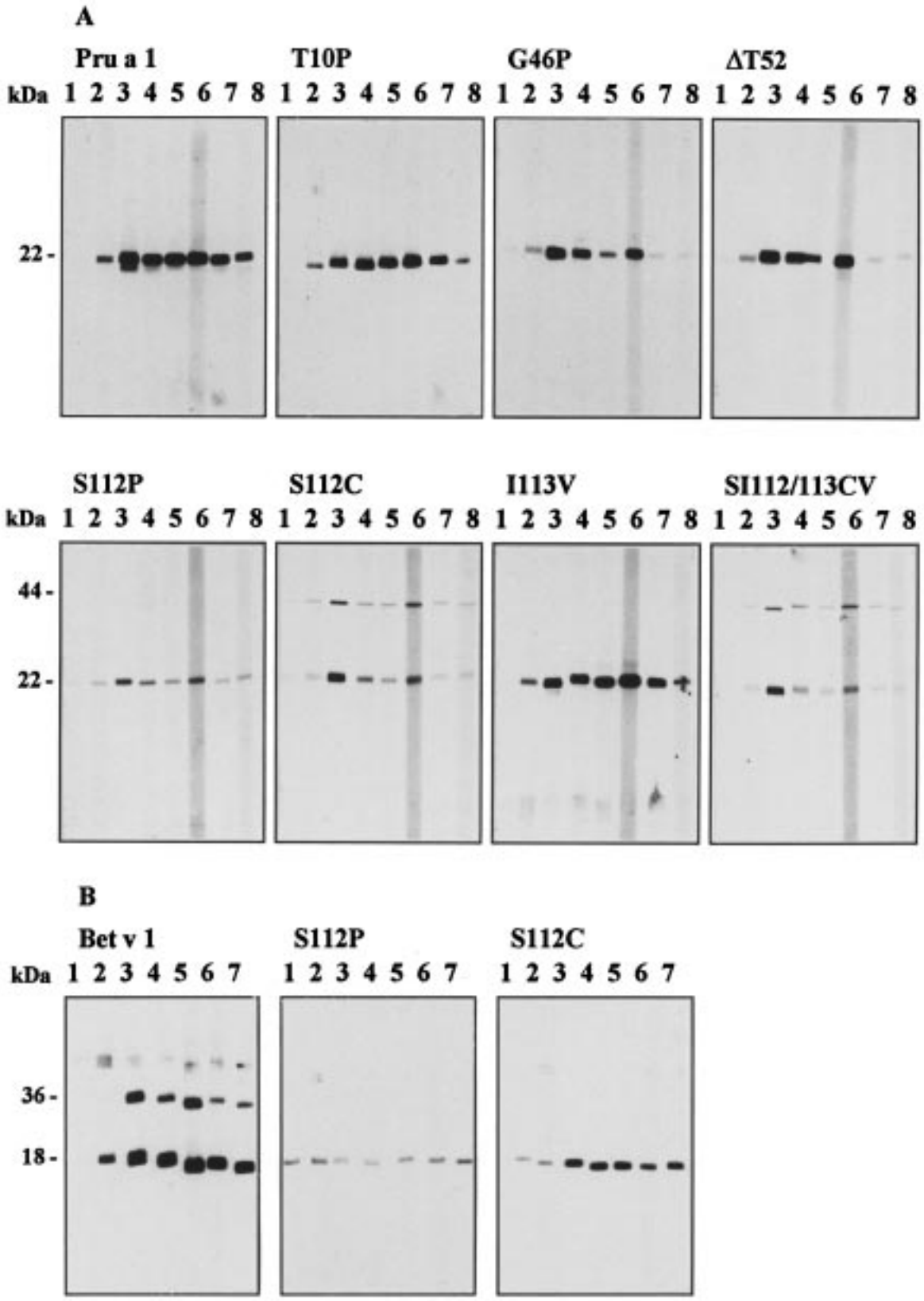

Fig. 7. IgE immunoblot of rPru a 1, rPru a 1 mutant fusion proteins T10P, G46P, ΔT52, S112P, S112C, I113 V, SI112/113CV (A) and rBet v 1a and the single point mutants Bet v 1 (S112P) and Bet v 1 (S112C) using patient sera (lanes 2–7: PEI 18, 50, 61, 62, 79, 82) and a serum from a non-allergic control PEI 22 (lane 1). For IgE immunoblot of rPru a 1 and rPru a 1 mutant fusion proteins an additional patient serum (Bo 111) was used (lane 8).

Table 2
Determination of specific IgE binding to Pru a 1, Bet v 1, mutant Pru a 1 and Bet v 1 proteins with seven patient sera and one non-allergic control (PEI 22) by EAST. Results are expressed as EAST classes

	EAST classes Pru a 1	T10P	G46P	ΔT52	S112P	S112C	I113 V	SI112/113CV	Bet v 1	S112P	S112C
PEI 22	0	0	0	0	0	0	0	0	0	0	0
PEI 18	2	1	0	1	1	1	1	0	3	0	2
PEI 50	4	3	3	3	2	2	3	3	4	0	4
PEI 61	3	3	2	3	0	0	3	1	4	0	4
PEI 62	3	2	2	2	1	2	3	1	4	0	4
PEI 79	3	3	2	3	0	0	3	0	4	0	3
PEI 82	3	2	0	0	0	1	2	2	4	2	3
Bo 111	2	2	0	0	0	0	1	0	4	0	3

evance of this position in Pru a 1 was also investigated. Moreover, the amino acid position (Ser112) of Bet v 1a, which is different in Bet v 1d was substituted by site-directed mutagenesis to investigate the importance of this position maintaining the reactivity of IgE epitopes (Fig. 7B). All proteins were expressed and purified as Histag-fusion proteins with a molecular mass of 22 kDa. No reduction of antibody binding was measured with Pru a 1 (T10P) where threonine in amino acid position 10 was changed to proline. For some patients, e.g. patients 7 and 8, a decreased IgE binding was observed when tested with the mutants Pru a 1 (ΔT52) and Pru a 1 (G46P). The single amino substitution of residue S112 to proline or cysteine efficiently reduced IgE binding to Pru a 1 for all patients tested, whereas the substitution of position 113 had no effect. Similar results were obtained with the Bet v 1 (S112P) and Bet v 1 (S112C) mutants. Substitution of amino acid residue 112 to proline prevented IgE binding, whereas the change in the same position to a cysteine only reduced the IgE binding for all patients tested. The dimeric Pru a 1 bands near 44 kDa, and the dimeric and tetrameric Bet v 1 bands, seen in Fig. 7A and B, are artifacts (Wellhausen et al., 1996 and unpublished results).

To confirm the observed differences in the IgE reactivity under native conditions, EAST class determinations were performed with sera from the seven cherry-allergic patients (Table 2). IgE binding was measured to allergen disks loaded with mutant Pru a 1 fusion protein and mutant Bet v 1 and wild type proteins, respectively. All sera presented IgE specific for Pru a 1 (EAST classes 2–4) and wild type Bet v 1 (EAST classes 3–4), whereas the control serum showed no IgE binding. A strong reduction of IgE binding was measured with both Pru a 1 mutant proteins bearing an amino acid substitution at position Ser112, resulting in EAST classes ranging from 0 to 1 for 6/7 sera for S112P and 5/7 sera for S112C. Reduction of EAST classes was also observed for all patient sera tested with the double mutant SI112/113CV protein. For three sera (PEI 18, PEI 82, Bo 111), no IgE binding was measured with the Pru a 1 proteins mutated in the P-loop region, except for PEI 18 with EAST class 1 in case of ΔT52. For amino acid positions Thr10 and Ile113 of Pru a 1, site-directed mutagenesis had no or little effect on antibody binding. Amino acid substitution of Bet v 1 Ser112 to proline completely prevented the IgE binding for 6/7 sera tested, whereas the substitution of the same residue to a cysteine had only a weak effect for 4/7 patient sera.

The allergenic potency of the mutant proteins rBet v 1 (S112P), rPru a 1 (S112P) was compared to rBet v 1 and rPru a 1 in the murine RBL-cell mediator release assay (Fig. 3). Maximal mediator release induced by cross-linking with Bet v 1 (S112P) was obtained at a protein concentration of 10 μg/ml, indicating an about thousendfold reduced reactivity of this rBet v 1 mutant. On the other hand, substitution of Pru a 1 with proline at this position reduced the allergenic poteny by factor 10 (maximal release at 100 μg/ml). Therefore, the amino acid substitution of Ser112 to proline drastically diminished the allergenic activity for both allergens investigated.

4. Discussion

Cross-reactivity studies and site-directed mutagenesis experiments were performed to identify key features that are crucial for the allergenicity of the investigated allergens.

4.1. Cross-reactivity of Pru a 1

IgE inhibition assays and the RBL-cell mediator release assays with purified Pru a 1, Mal d 1, Api g 1 and Bet v 1 were carried out in a dose dependent manner. The results clearly confirmed the cross-reactivity between all investigated food allergens and Bet v 1. Cross-inhibition experiments between Api g 1 and Pru a 1, and additional IgE-inhibition studies with Bet v 1

and Mal d 1 were performed in the EAST. Api g 1 and Pru a 1 were highly reactive with anti-Bet v 1-IgE antibodies, as demonstrated by the complete inhibition of IgE binding to the food protein by extremely low amounts of Bet v 1. With both Pru a 1 and Api g 1 at the solid phase, the inhibition potency of Bet v 1 was several orders of magnitude higher than the self inhibition by the food allergens. The same observation has been made with Mal d 1 (Vieths et al., 1995) and with Api g 1 (Breiteneder et al., 1995) by immunoblot inhibition. Finally, the inhibition experiments and the EAST class determinations showed a reduced affinity of the cross-reactive IgE to the food allergens when compared to Bet v 1. We can also presume minor epitope differences between Pru a 1 and Mal d 1, since Mal d 1 inhibited the IgE binding to Pru a 1 up to 50% at the maximal concentration. Although all three sera had a clearly positive EAST to both, Pru a 1 and Api g 1, the results revealed the presence of different epitopes between the fruit (Pru a 1, Mal d 1) and vegetable (Api g 1) allergens, which were confirmed by IgE-immunoblot inhibition experiments. No cross-reactivity between Pru a 1 or Mal d 1 and Api g 1 was measured. This phenomenon could be explained by the existence of different cross-reactive epitopes on Bet v 1, with a subset cross-reacting with fruit proteins and another subset cross-reactive with celery and possibly carrot. Interestingly, no differences between the two patient groups, allergic to one allergen or allergic to both the cherry and the celery allergen, could be observed in the EAST inhibition studies. The RBL cell mediator release assay with murine anti-birch pollen IgE revealed roughly identical allergenic potencies for the three fruit allergens Pru a 1, Mal d 1 and Pyr c 1. The allergenic potency of Api g 1 was reduced by a factor of 10 in comparison to the fruit allergens. In comparison to Bet v 1 the reactivity of the investigated food allergens was diminished a hundredfold, whereas the reactivity of Api g 1 was reduced by a factor of 10^3. Since cross-linking of anti-birch pollen IgE by all investigated allergens clearly induced a significant mediator release, these results correspond well with the known link between birch pollinosis and oral allergy syndrome (Vieths, 1997).

4.2. Epitope analysis

Structural investigations were in concordance with the results of the cross-reactivity studies. For most of the secondary structure elements of Bet v 1 the structure prediction was in accordance with the experimentally obtained structure (Gajhede et al., 1996). Since an almost identical secondary structure was predicted for all four food allergens we can assume a similar secondary structure for these birch pollen related allergens. The prediction revealed no indication for the experimentally observed weak cross-reactivity between Pru a 1 and Api g 1. For Pru a 1 the secondary structure was investigated by CD-spectroscopy. The results revealed further evidence for identical secondary structure elements in both the pollen and the fruit allergen. The model of the tertiary structure of Pru a 1, created with the structure of Bet v 1 as a template, presented an overall identical structure with minor differences in the tertiary fold.

Thus, the different allergenicity of these two proteins is probably due to key residues influencing the antigenic determinants. To prove this hypothesis, the IgE reacitvity of allergen fragments was investigated and site-directed mutagenesis experiments were performed. The very weak IgE reactivity of the fragments indicated that the IgE binding to Pru a 1 strongly depends on the integrity of the structure of the whole molecule. Therefore, amino acids were selected for site-directed mutagenesis by comparison of high and low IgE binding allergens. Mutation of the P-loop region lead to a reduced, but not to a complete depletion of IgE binding from several tested sera indicating the possibility that this region directly participates in an IgE epitope. Recently, this region was proposed as an antibody binding domain by (Spangfort et al., 1997). Furthermore, site-directed mutagenesis of Pru a 1 and Bet v 1 at serine 112 caused a nearly complete loss of IgE binding, in particular, when proline was introduced at this position. It is well known that the amino exchange to proline can drastically affect the protein structure and the complete loss of allergenicity can only be explained by an altered tertiary structure, as all epitopes responsible for binding and crosslinking of specific IgE were destroyed by a single point mutation. Dimeric and tetrameric structures of Pru a 1 (S112C and SI112/113CV) and of wild-type Bet v 1 were detected by immunoblotting. Since the Pru a 1 mutants had not been alkylyted after reduction, this phenomenon could be explained by an inter-molecular disulphide bond. However, Bet v 1a contains no cysteine. Since dimeric Bet v 1 was identified on immunoblot of pure natural Bet v 1 even under reducing conditions (Wellhausen et al., 1996) and was also detected in recombinant material (own unpublished data), covalent inter-molecular linkages different from S–S-bonds appear to be responsible for this problem. Initial CD-spectra of the non-allergenic Pru a 1 variant indeed indicated an altered structure in comparison to the wild type protein (not shown). Recently, a Bet v 1 multiple mutant protein including an exchange of amino acid serine 112 with weak IgE reactivity and intact tertiary structure was described (Ferreira et al., 1998). The discrepancy concerning the correct protein folding may be explained by the substitution to a cysteine instead of proline in the described Bet v 1 mutant. According to the results of Ferreira et al.

(1998) our single point Bet v 1 mutant S112C also showed a slightly reduced IgE binding capacity for 4/7 patient sera (as shown by the EAST results). In summary, we have shown that cross-reactive epitopes on Bet v 1 and related food allergens can be destroyed by single point mutations of amino acid residues in identical positions. In assumption that the structural changes are stable, meaning that refolding to the natural conformation is excluded, and that these mutants show a preserved T cell reactivity, such variants can probably be used for a safer specific immunotherapy of birch pollinosis and related food allergy.

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