

The Major Human Structural IgE Epitope of the Brazil Nut Allergen Ber e 1: A Chimaeric and Protein Microarray Approach

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A protein microarray system containing different dilutions of 77 related and non-related proteins was used to show that IgE from subjects allergic to Brazil nut specifically recognise the seed 2S albumin protein (Ber e 1). Further, correctly folded chimaeric 2S albumin proteins containing structural epitope replacement were constructed and directed to the secretion pathway of the methylotropic yeast *Pichia pastoris*. Through the use of a chimaeric protein microarray system together with sera from a panel of 18 well-characterised Brazil nut allergic subjects, a structural IgE epitope of Ber e 1 was mapped to a helix-loop-helix region. The same structural region has been previously reported as the immunodominant region in related food allergens by different techniques. In conclusion, the combination of chimaeric proteins and protein microarrays will greatly facilitate the screening of a large number of individuals for a particular structural epitope and help to further our understanding of how proteins are recognised by the adaptive immune system.

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Keywords: structural epitope; 2S albumin; prolamin superfamily; food allergen; protein microarray

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Introduction

The plant seed 2S albumins belong to the large prolamin superfamily of proteins that are characterised by their conserved antiparallel bundle of four α -helices held together by four disulphide bonds in a distinctive right-handed fold.¹ NMR constraint maps of the disulphide patterns are available for a few representative proteins of this class and are in perfect agreement with the disulphide pattern assigned by mass spectrometry and high-resolution crystal structure data.² A large number of known food allergens such as plant

α -amylase/trypsin inhibitors, 2S albumin and lipid transfer proteins (LTPs) belong to the prolamin superfamily and may have evolved from the same ancient gene.¹ As a class, the 2S albumins are water-soluble proteins, products of multi-gene families and are present in seeds of a wide range of dicotyledonous and monocotyledonous species.³ Although some functions have been assigned to some 2S albumins, their specific biological roles are mostly unknown.³

Elsewhere, we described the production and biochemical characterisation of Ber e 1, the major 2S albumin allergen from Brazil nut (*Bertoletia excelsa*).⁴ We showed that Ber e 1 and SFA8, the 2S albumin from sunflower seed, are secreted at high levels by the methylotrophic yeast *Pichia pastoris* and that the recombinant proteins mimic their native counterparts in structure and biophysical characteristics. Subsequent studies demonstrated that both proteins were highly resistant to thermal and chemical-induced denaturation and proteolytic digestion, important attributes in the assessment of intrinsic allergenicity of proteins.^{5,6} Our results have suggested Ber e 1 to be the only intact Brazil nut seed protein that withstands peptic digestion,

Abbreviations used: LTPs, lipid transfer proteins; ER, endoplasmic reticulum; UPR, unfolded protein response; ELISA, enzyme-linked immunosorbent assay; TMB, 3,3',5,5'-tetramethylbenzidine; Ig, immunoglobulin; BSA, bovine serum albumin; MMD, minimal medium dextrose; MMG, minimal medium glycerol; MMM, minimal medium methanol; PVDF, polyvinylidene fluoride; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitro tetrazolium blue chloride; SGSG, Ser-Gly-Ser-Gly.

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making it a prime candidate for recognition by the gut immune system.

Although the stability and abundance of Ber e 1 are likely to contribute to its allergenic nature, the widespread consumption of other stable 2S albumins suggest that the stability is only one of a number of factors important in determining the intrinsic allergenicity of protein.⁷

In order to understand how the immune system recognises those proteins and further our understanding of the attributes that make an allergen, we have produced properly folded chimeric proteins by replacing the structural domains from SFA8, a widely consumed and closely related protein, with the domains from Ber e 1. Using a panel of 18 Brazil nut-specific allergic subjects and a chimeric protein microarray format, we have identified the structural immunodominant region of Ber e 1. Other studies using hybrid proteins have been reported recently,^{8,9} however, we show here that the combination of chimeric protein design in an eukaryotic system and microarray screening is a promising platform for generating large numbers of structural epitopes.

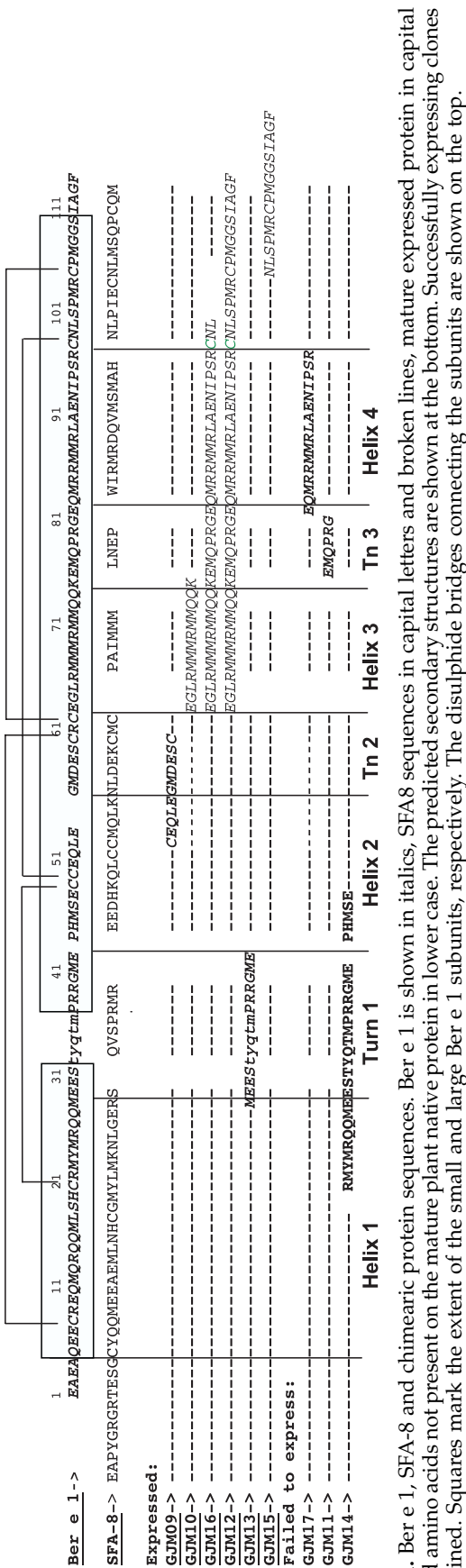
The ability to screen a large number of individuals and identify conformational or discontinuous epitopes that depend on the native conformation of the protein is an encouraging first step towards developing hypo-allergenic variants for immunotherapy.¹⁰

Results and Discussion

Design and expression of chimaeric proteins

Although the complete 3D structure for neither Ber e 1 nor SFA8 is available, in designing the chimaeric proteins we exploited our own and published molecular models of their structures^{4,11} which share key secondary structural features, as indicated in Figure 1. During the initial design, attention was given to the replacement of 50% (GJM12) of the sequence and to the replacement of all turn structures aiming at minimising potential protein misfolding during protein expression at both small and large subunits (GJM 09,11,14). As the disulphide mappings of Ber e 1 and SFA8 have been determined experimentally and are conserved throughout the 2S family of protein,^{2,4,11} the chimaeric replacements were anchored, whenever possible, to the cysteine residues involved in the disulphide bridges (Figure 1). After initial analysis further designs (GJM 17,16,10 and 13) were attempted in order to narrow down to the conformational epitope region.

We have shown here and elsewhere⁴ that the recombinant Ber e 1 and SFA8 albumins can be secreted from *P. pastoris* in sufficient yields to facilitate structural and functional analyses. Furthermore, we have shown that the purified recombinant proteins have secondary structures identical with those of the purified native proteins.^{4,5}



However, as indicated in Figure 1, not all chimaeric proteins were successfully expressed. Of the nine chimaeras constructed at the gene level, only six were expressed in sufficient yield to be useful in further studies. Although attempts have been made to minimise the potential problems during expression, the absence of expression was not completely unexpected. We know for instance, from our own unpublished studies of *P. pastoris* and those from others in *Saccharomyces cerevisiae*,^{12–14} that the secreted yields of proteins can depend upon the stability of the protein, i.e. more stable structures, measured in most cases by higher unfolding temperature, can lead to a higher secreted yield. The generality of this observation has been confirmed in several systems.¹⁵ It is therefore possible that some of the chimaeric proteins were intrinsically unstable, suggesting that some important interactions between particular amino acids had been compromised.

The successful secretion of proteins from eukaryotes is highly dependent upon the correct fold being achieved during passage through the secretory system. Assisted folding of secreted proteins occurs in the lumen of the endoplasmic reticulum (ER) and passage beyond this point is subject to satisfying the scrutiny of the cell's innate quality control system.^{15,16} Those proteins that are incompletely folded, and retain exposed hydrophobic areas, initiate the unfolded protein response (UPR) which results in the retro-translocation of proteins from the ER to the cytosol where degradation by the proteasome ensues.^{17–19} The secretion achieved with the *P. pastoris* expression system is therefore exploited to provide quality control of expressed proteins. The results showed that the six secreted chimaeric proteins (Figure 2) have therefore achieved a fold *in vivo* that enabled their secretion.

Protein processing and folding

All chimaeric plasmids described here were constructed to include the pre-pro region of the *S. cerevisiae* α -mating factor that directs the proteins to the ER and therefore to the secretion pathway. The mechanism for removal of the pro-sequences is

well understood and occurs *via* endo-proteolytic cleavage by the KEX2 protease in the Golgi, releasing the mature protein.^{20–22} In previous work we have inferred that during the secretion of plant 2S albumins, other protease families might be involved in the final protein processing.²³ The same processing occurs *in planta*. With rare exceptions, most of the 2S albumins found in seeds are encountered as two subunits in which the exposed loop regions have been processed during their trafficking to the protein storage bodies.

It is clear from the various band sizes in Figure 2 that further cleavage events described above have also occurred within the chimaeric constructs.

In order to confirm whether the secondary structures of the chimaeric proteins have been affected during protein expression, a comparative CD analysis was carried out. Figure 3 shows that the five chimaeric proteins have similar α -helical secondary structure content of their native counterparts. The 50% construct (GJM12), however, has shown a great degree of instability in solution that was reflected in the pattern of antibody recognition as discussed below.

Protein microarray

The use of the purified allergens printed as microarrays on pre-activated glass slides has been described extensively elsewhere.^{24,25} There are several advantages of using such an approach instead of the conventional Western-blotting system or ELISAs: (i) the proteins do not need to be heat-denatured before a gel separation; (ii) no SDS or other detergent-bound protein is present; (iii) all the proteins are represented at similar concentration or at a known concentration; (iv) a small amount of protein is required; (v) a small amount of serum is used in each experiment; (vi) more than one Ig class can be detected simultaneously; (vii) appropriate controls such as human IgG and negative controls such as total IgG and human albumin can also be monitored simultaneously.

In order to test the immunoreactivity of the chimaeric proteins, a range of protein preparations and dilutions were covalently bound to a glass surface and tested against specific allergic subject sera. All the necessary control experiments without the human sera or without the secondary biotinylated antibody and the streptavidin dye were performed and have not shown any significant level of detection. Further, sera from soybean allergic and non-atopic subjects did not show any binding to either Brazil nut total protein or Ber e 1 (results not shown).

During initial experiments, high protein concentrations were attempted in order to avoid the saturation effect caused by excess of cross-reactive IgG in the sera. However, as shown in Figure 4, it was clear that concentrations higher than 1 mg/ml of Ber e 1 per spot would not be necessary. Various sera dilutions (1/4, 1/10, 1/32, and 1/64) have been used and larger IgE recognition qualitative

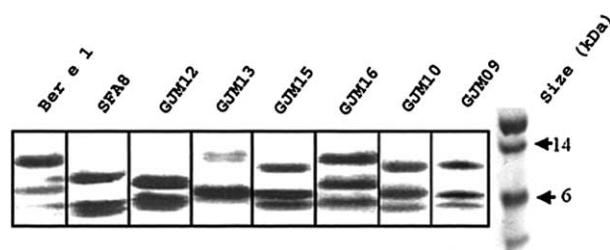


Figure 2. Reducing SDS-PAGE of expressed recombinant proteins. The different post-translational processings of the reverse-phase purified recombinant proteins are shown in reducing SDS conditions. Batch-to-batch variability of the amount of the large unprocessed band was observed.

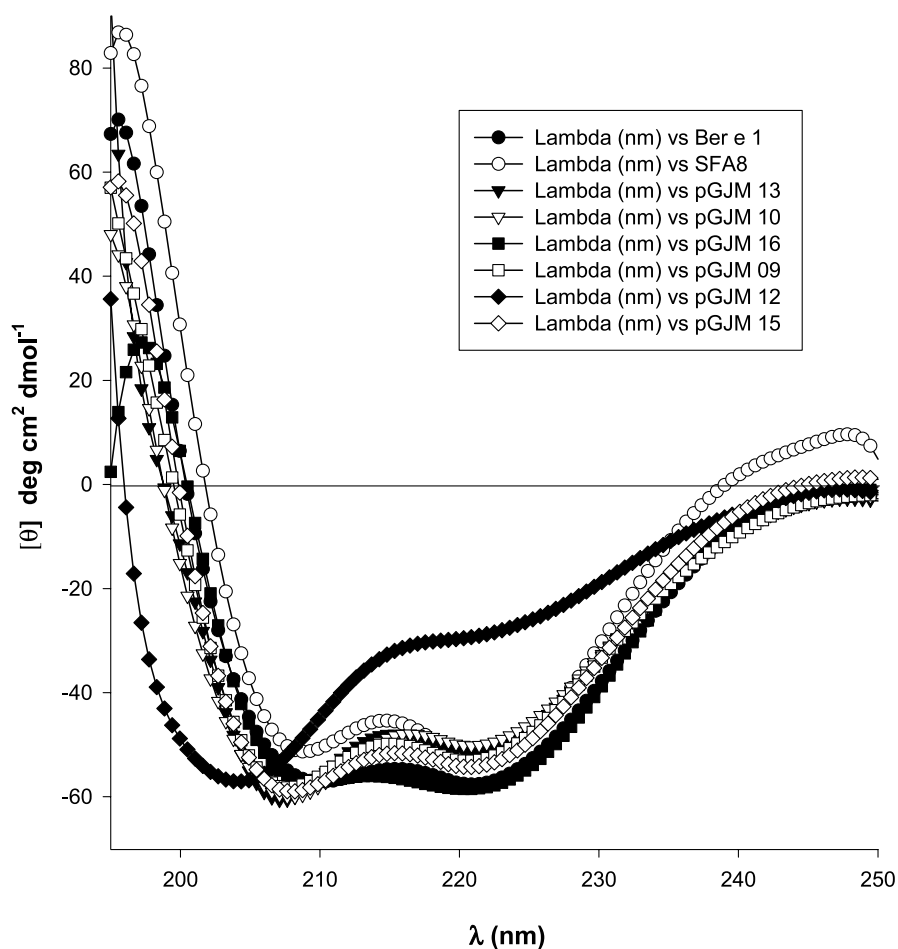


Figure 3. CD spectra in the far-UV region of recombinant GJM clones at pH 6.8 and 25 °C.

variations were observed at higher dilution, particularly when different blood donors were compared (results not shown). Similar to the published literature, the IgE microarray results presented here were variable but reproducible. A more complete statistical analysis regarding reproducibility, intra and inter batch variation with allergic-specific donors other than to Brazil nut will be published elsewhere (unpublished results).

Regarding the specificity of the binding of the IgE

two kinds of responses have been observed. As exemplified in Figure 5 for the IoW37 subject, this patient has shown a typical specific IgE recognition towards Brazil nut total proteins and in particular towards Ber e 1. The same pattern of specificity was found in 17 out of 18 Brazil nut-specific sera tested. Interestingly in this large group, the IgG4 isotype did not recognise Ber e 1 but quite often cross-reacted with proteins from chicken origin (Figure 5). A different and interesting pattern of recognition

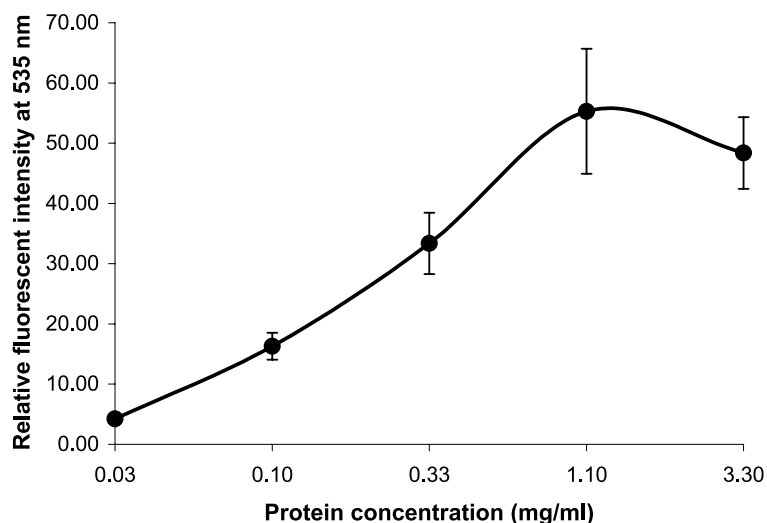


Figure 4. IgE detection showing the saturation effect of increasing concentration of Ber e 1 on the glass slide surface. The specific immune serum from the IoW37 subject was used in this experiment. Error bars are standard deviations from five determinations.

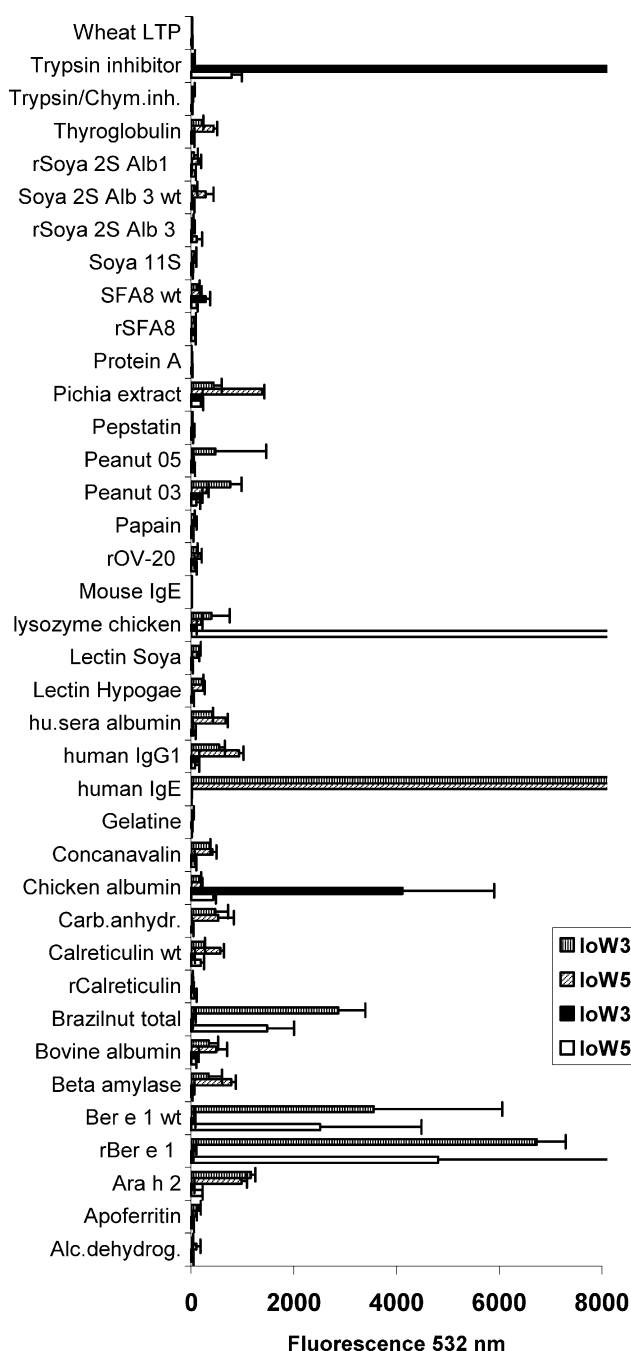


Figure 5. Total protein microarray. Sera from Brazil nut allergic patients diluted 1:10 in PBS. Multiple detection for IgE and IgG4 as indicated in the legend. Results and standard deviation are averages from five determinations.

was shown by the IoW52 serum. This serum when analysed showed a class 2 RAST and no significant levels of IgE binding to Ber e 1 using the microarray. However, it showed a specific IgG4 recognition to Ber e 1. This is the only serum for which this pattern was observed. The results suggest, but do not prove, that this particular subject is either becoming allergic to Brazil nut proteins or outgrowing his/her allergy, most probably the latter.

The specific IgE recognition of the major allergen Ber e 1 by subjects allergic to Brazil nut has been described using other techniques such as Western blot²⁶ and ELISAs.⁵ In order to validate the protein microarray system, the same serum samples described in both experiments were used in the present work. In order to further characterise the

nature of the IgE recognition, competition micro-ELISAs were set up using Ber e 1, the related 2S albumin from sunflower (SFA8) and bovine serum albumen (BSA) (Figure 6). Only Ber e 1 protein was able to displace the IgE binding reaction. Taken together these results have shown that the sera used mainly recognised Ber e 1 and that the binding epitope of Ber e 1 is available for IgE in the microarray format.

Epitope-specific microarray

In order to map the IgE immunodominant region of Ber e 1, the chimaeric proteins Ber e 1×SFA8 were immobilized on the microarray glass slide and screened against the same panel of sera from

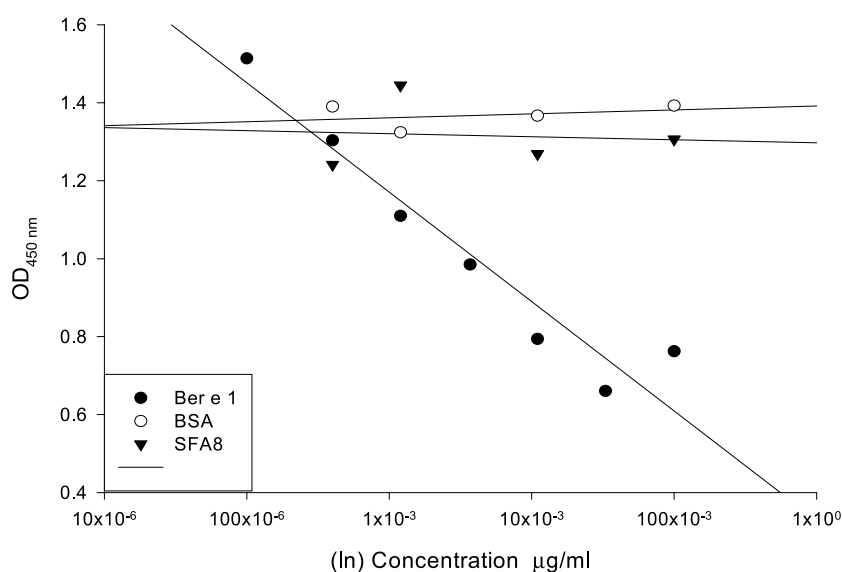


Figure 6. Competitive micro-ELISA. Human IgE-specific ELISA using Brazil nut-specific sera diluted 1:4 (IoW37 subject from Figure 4) in competition with increasing concentration of bovine serum albumin, SFA8 and Ber e 1.

specific Brazil nut allergic subjects described above (Figure 7). Despite the polyclonal nature, the different sera showed a specific response to the GJM16/10 region and some towards GJM 13 but reduced binding to GJM15/09 and SFA8. The results shown in Figure 7 were obtained with the spots plotted at concentrations of 1 mg/ml of protein and the high background binding of the ManD serum to SFA8 was not observed at lower protein concentration. GJM16 corresponds to the sequence EGLRMMMRMMQQKEMQPRGEQMR RMMRLAENIPSRCNL that comprises a region predicted to take a helix3-turn4-helix4 conformation. The binding to the small helical region of GJM10 further confirmed this region of the Ber e 1 as the major structural IgE immunodominant region for this allergen. All efforts to narrow down the region to a smaller epitope in turn 4 have resulted in non-expressing clones. Interestingly, the 50% replacement region represented by GJM12, which includes most of the helix3-turn4-helix4 and N-terminal region, did show a comparatively reduced IgE binding. It is clear from the CD results that some major conformational alterations have taken place with GMJ12. Preliminary work with GJM12 suggests that the recombinant protein is produced in the correct fold but changes its conformation during the purification procedure. The reasons why this “unstable” clone can be secreted in *Pichia*, and detailed kinetics studies with GMJ12, might be subjects for future work. However, what these results stress is how sensitive the IgE epitope of Ber e 1 is to conformational changes.

The region displayed by GJM13 comprises turn 1. Most of the post-translational processing that takes place on the native Ber e 1 seed protein occurs at this region. Preliminary data indicate that the O-glycosylation reported by Alcocer *et al.*⁴ occurs in the serine/threonine residues also contained within this region. Hence, our results cannot exclude the possibility that the polyclonal IgE binding observed to the GJM13 region is not protein-specific but due

to glycosylation. Work is on-going to clarify the role of processing/glycosylation on the IgE binding.

Overall, the mapping of the helix-loop-helix region of Ber e 1 presented here is in agreement with similar major epitope regions identified in three other 2S albumin allergens Jug r 1,²⁷ Bra J 1e and Sin a 1^{28,29} using different methodologies. In the case of Jug r 1, the linear IgE epitope was determined by peptide overlapping sequences and the loss of IgE binding confirmed by point mutations.²⁷ In the case of Bra j 1e and Sin a 1, a common epitope was detected in the large chain by means of cross-reactivity with a specific monoclonal antibody.^{29,30}

Detection of IgE to specific linear peptides

In order to compare the information obtained by the chimeric protein/microarray system with the conventional linear epitope approach, sera from allergic-specific Brazil nut subjects, IoW37 and the Manchester Saint Mary's patients, were screened against 15 overlapping peptides encompassing the entire Ber e 1 mature protein sequence (Figure 8). An initial comparison of IgE binding to the overlapping peptides and Ber e 1 showed that the signal to the folded Ber e 1 was at least fourfold higher.

Only one linear peptide derived from the Ber e 1 sequence bound IgE antibodies consistently by both ELISA (Figure 8a) and dot-blot assay (Figure 8a, inset). This sequence, from the second peptide (QMQRQQMLSHCRMY), starts at amino acid 11 within helix 1 of the Ber e 1 small subunit (Figure 1). As we do not possess chimeric constructs encompassing the same region, it becomes difficult to assess whether this is a true binder or an artifact of the technique. However, Western blot results have clearly shown that IgE antibodies recognise primarily the reduced and SDS-linearised Ber e 1 large subunit chain (results not shown) that does not contain such peptide sequence.

Overall the pattern of peptide recognition of Ber e 1 differs markedly from the binding of IgE to

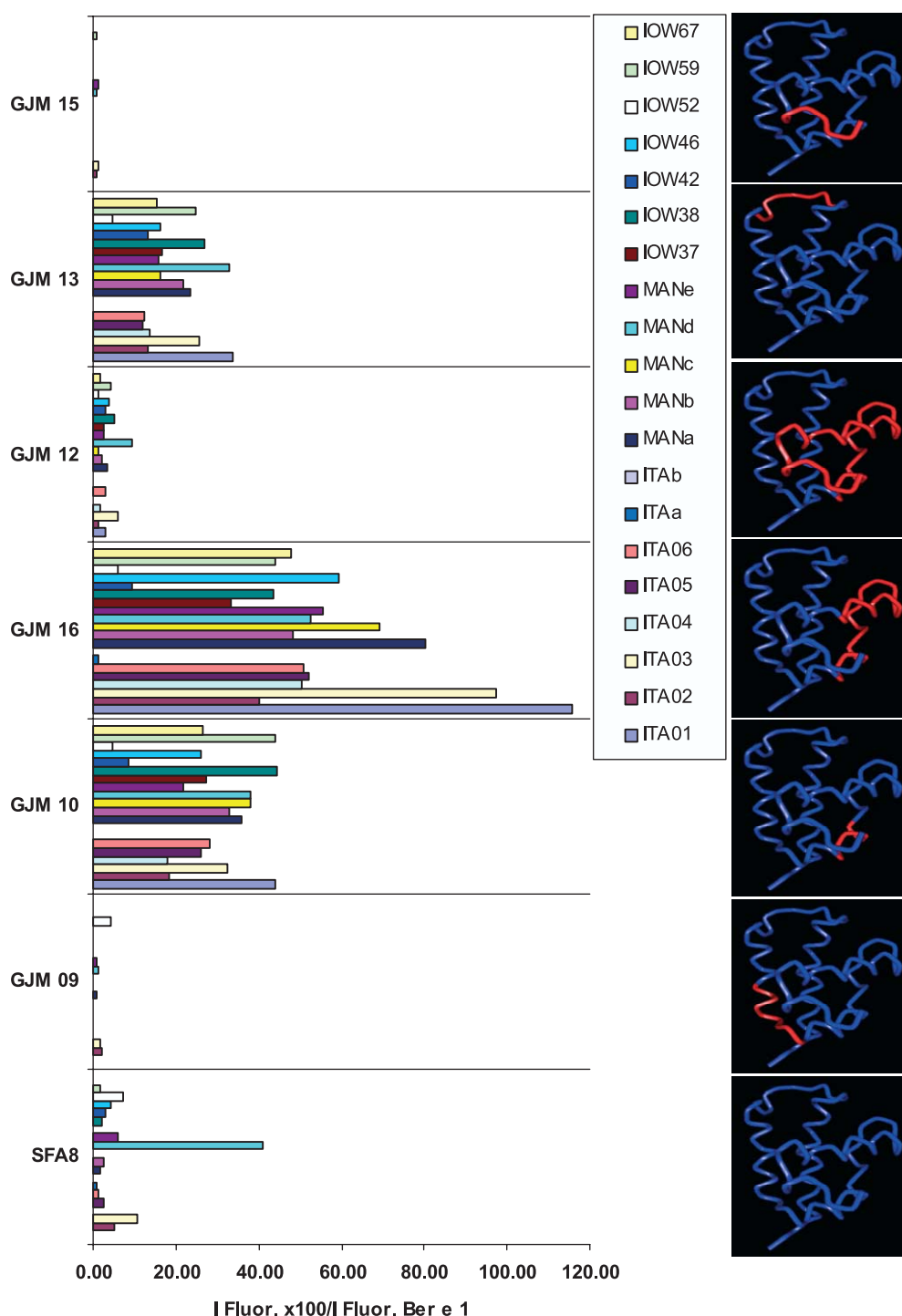


Figure 7. IgE binding specificity of 18 Brazil nut allergic subjects to the recombinant chimeric proteins Ber e 1 x SFA8. The fluorescence readout is shown as the percentage of the total fluorescence observed with intact Ber e 1. ITAa and b are subjects not allergic to Brazil nut (negative controls). Results are the average from five determinations.

equivalent linear peptides from peanut Ara h 2, a putative 2S albumin from peanuts. Sera reactive to peanut by ImmunoCAP[®] were found to bind consistently up to ten sites on Ara h 2 by ourselves and others.³¹ In addition, one monoclonal antibody raised to partially purified Ara h 2 also reacted very strongly with a short Ara h 2 linear peptide (unpublished results).

In contrast to Ara h 2, the corresponding linear sequence of Ber e 1 appears to be immunologically of

minor importance. From the limited number of patients studied, it is not possible to exclude that additional linear IgE epitopes on Ber e 1 may act as minor determinants in some individuals sensitive to Brazil nut. Recently one serum, from a patient with a long-standing Brazil nut allergy, also recognised amino acid residues 60–73 (CRCEGLRMMMMRMQ) of peptide 9 by ELISA (Figure 8b) and corresponding to the large subunit (Figure 8b), agreeing in this instance with the chimaeric protein approach.

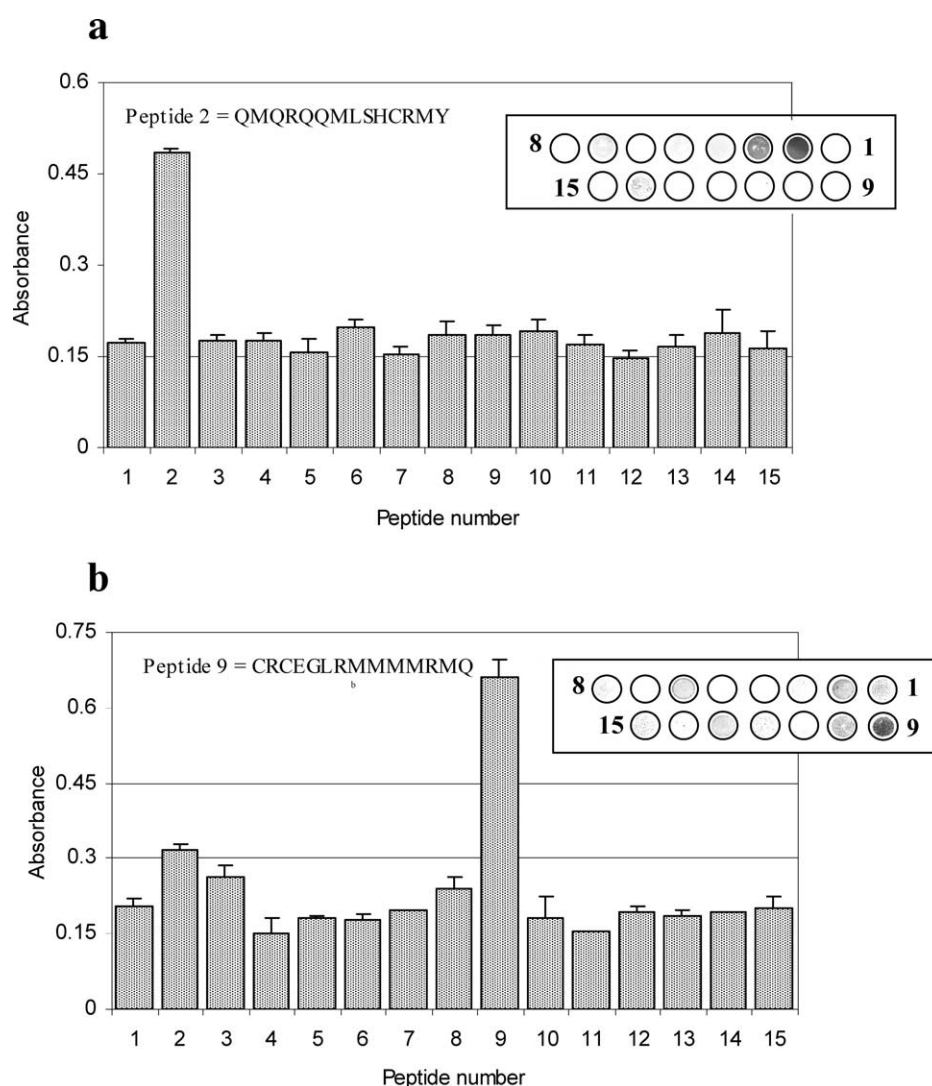


Figure 8. Fifteen 14 amino acid peptides offset by seven, were synthesised covering amino acid residues 36–146 of Ber e 1. Peptides 1–4 span the small subunit and 5–15 the large subunit. The cumulative ELISA results for seven patients with Brazil nut-specific IgE consistently bound peptide 2 within the small subunit (a); confirmatory analysis by dot-blot of a representative serum is shown in the inset in a. Additional binding in ELISA to peptide 9 on the large subunit was detected in one patient with long-standing Brazil nut allergy (b). Dot-blot analysis is shown in the inset in b. This serum weakly recognized the linear sequences in peptide 6 (RRGMEPHMSECCEQ) and 13 (MRLAENIPSRCNLS) not detected by ELISA. Marks above the bars indicate standard errors. The IgE binding to peptides was only around one-quarter of the signal for Ber e 1 (data not plotted).

Nevertheless, the cumulative results demonstrate that in the majority of Brazil nut-sensitive individuals, Ber e 1-specific IgE is predominantly directed against either longer or non-linear binding sites. With the exception of the one patient shown, there was no overall agreement between the IgE linear epitope in the small 2S subunit and the conformational epitope located at the large 2S subunit.

Conclusions

Our results show that chimaeric proteins with structural epitopes replaced by regions from other proteins can be expressed and secreted by the methylotrophic yeast *P. pastoris*. Using a protein microarray system we have shown that subjects with allergy to Brazil nut produce IgE specific to Ber e 1 protein

against a large background of other related and non-related proteins. Using the chimaeric proteins and the protein microarray system we have mapped the major structural epitope determinant of Ber e 1. Using peptides, the linear epitope of Ber e 1 has also been determined and, in contrast to the peanut Ara h 2, the linear epitope peptide of Ber e 1 appears to be immunologically of minor importance. The combination of chimaeric proteins and microarrays will greatly facilitate the screening of a large number of individuals for a particular structural epitope.

Materials and Methods

Patients

Sera from three groups of patients that were fully

characterised and described.^{5,26} were used in the present study. The first group comprises sera from six Italian outpatients who reported clinical reactions to ingestion of Brazil nut, with documented history of anaphylactic shock and positive CAP system results.²⁶ The second group comprises sera from seven UK outpatients from The David Hide Asthma and Allergy Research Centre, St Mary's Hospital, Isle of Wight, whose allergy to Brazil nut was confirmed by skin prick test, RAST, and labial/oral challenge.⁵ The third group comprises sera selected from an anonymised registered research database at St Mary's Hospital, Manchester, UK. These sera were reactive to Brazil nut as judged by use of the Pharmacia ImmunoCAP[®] system at levels >3kU_A/l.

Proteins

Seventy-four purified protein and protein extracts were used in the construction of the array. They were: chicken albumin, bovine albumin, chicken lysozyme, alcohol dehydrogenase, trypsin inhibitor, thyroglobulin, apoferritin, β -amylase, gelatine, gluten, carbonic anhydrase, protein A, pepstatin, papain, concanavalin, soya lectin, peanut lectin, and soya trypsin-chymotrypsin inhibitor, all from Sigma (Sigma-Aldrich Co. UK); the soya 11S fraction and wheat LTP were donated by Dr Clare Mills from the Institute of Food Research, Norwich, UK; the nematode calreticulin samples were donated by Professor David Prichard from the School of Pharmacy, University of Nottingham; control human and mouse IgE were donated by Dr Richard Pleass from the School of Biology, University of Nottingham; and the following proteins were prepared in *P. pastoris* at our lab: soybean 2S albumins A11 and A13,²³ sunflower SFA-8 2S albumin, Brazil nut 2S albumin (Ber e 1), chimeric Ber e 1 $\times$ SFA-8, *Arabidopsis thaliana* 2S albumin, LTP from pears, human lysozyme, nematode proteins Ov-20 and ABA-1, and 22 SDS-PAGE preparative fractions of peanut seed proteins. The grid positioning of the protein array was earmarked with Streptavidin-Cy3 and Cy5 (Amersham, UK).

Media composition for *P. pastoris*

P. pastoris GS115 (Invitrogen) was used throughout this work. The growth conditions for maintenance, enrichment and induction have been described.⁴

Construction of expression plasmids

The construction of both plasmids containing the cDNA sequence of sunflower albumin 8 and Ber e 1 have been described.⁴ The chimaeric plasmids containing the sunflower sequence background and the Ber e 1 replacement sequence were assembled by a two-step procedure: splice over extension polymerase chain reaction (SOE)-PCR.³² Essentially, the two 2S albumin gene fragments were amplified by PCR using specific primers carrying the overlap region. The PCR reaction was run for 35 cycles of 94 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 30 seconds, using Amplitaq (two units/100 μ l; Applied Biosystems) according to the manufacturer's instructions. The PCR products were gel-purified (Qia-prep; Qiagen) also according to the manufacturer's instructions and small aliquots (1–2 μ l) used for the second round of amplification using external primers. During some PCR amplifications, more than one product was generated. In order to optimise the PCR condition for the desired product, a commercial PCR Optimization Kit (Sigma) was used following the manufacturer's

instruction. The final PCR products (ca 400 bp) were then gel-purified, cloned and sequenced as described.⁴

Transformation and expression in *P. pastoris*

pPIC9-derived plasmids containing the 2S protein-encoding sequences were transformed into *P. pastoris* GS115 and selected on MD plates following electroporation according to the manufacturer's instructions (Invitrogen). Clones able to grow in medium lacking histidine were selected, expanded, induced with methanol and higher protein expresser clones were identified by dot-blot screening as described.⁴

Large-scale protein production and purification

Selected clones were grown at high density ($A_{600} > 3$) in five 4 l-flasks containing minimal medium glycerol (MMG), induced in minimal medium methanol (MMM) and the 2S albumin proteins purified by heparin-Sepharose as described.⁴ The purified 2S proteins were further purified by HPLC using a preparative stainless steel C8 reverse phase column (300 Å, 5 μ m, 100 mm $\times$ 10 mm; Varian Ltd, Surrey, UK) and guard column (300 Å, 10 μ m, 10 mm $\times$ 10 mm) at 2 ml/minute, 0–100% water/acetonitrile gradient. The eluted peak from the reverse phase column was then concentrated under vacuum for four hours at room temperature and freeze-dried overnight.

MicroELISAs

Maxisorp[®] plates (384 wells) were coated with 100 ng of protein/ml in phosphate-buffered saline (PBS) (10 μ l/well) overnight at 4 °C, blocked with 3% (w/v) BSA in PBST (PBS plus 0.1% (w/v) Tween 20) (30 μ l/well) for 30 minutes at 37 °C, and incubated with serum diluted 1:10 (20 μ l/well) for two hours at 37 °C. Specific IgE detection was carried out with goat biotinylated anti-human IgE (Vector lab. Inc, Burlingame, USA) diluted 1:250 (v/v) in PBST (20 μ l/well) for two hours. For visualization, streptavidin-horseradish peroxidase (Sero-tec, UK) diluted 1:1000 (20 μ l/well) was applied using 3,3',5,5'-tetramethylbenzidine (20 μ l/well) for 20–30 minutes at room temperature as substrate. The reaction was stopped with 2.5 M H₂SO₄ (10 μ l/well) and the absorbance measured at 450 nm.

Overlapping linear peptides

A series of 15 overlapping peptides covering amino acid residues 36–146 from the Ber e 1 primary sequence (SwissProt P04403) were prepared by Mimotopes[®]. Individual linear peptides are 14 amino acid residues long and subsequent peptides are offset by seven amino acid residues. Peptides 1 to 5 correspond to the small Ber e 1 subunit and the remaining ten to the major subunit. To correctly orientate the peptides on the microtitre plate they were conjugated with biotin, *via* an SGSG spacer. The efficiency of biotinylation was confirmed by Mimotopes[®] analysing two control peptides synthesised at the same time. No molecular ions were detected, consistent with a deletion of biotin in either of the two control samples studies. This confirms that all the target peptides present in the sample were biotinylated. Individual peptides were initially dissolved in 50% (v/v) acetonitrile/water and the mixture sonicated for ten minutes before freezing at –20 °C.

Detection of IgE to specific linear peptides

Microtitre wells (Immulon 2; Dynex) were coated overnight with 1 µg/ml of recombinant streptavidin (Sigma; S-0677) in PBS (pH 7.4). Following coating, streptavidin was replaced with 1% (w/v) fish gelatine (Sigma; G-7041) in PBS and the plates blocked for one hour with shaking at room temperature. Plates were washed three times in PBST and blotted dry. Each peptide, pre-diluted to 5 µg/ml in PBST, or biotinylated recombinant Ber e 1 when required, was added to the streptavidin-coated wells and the plates again incubated for one hour with shaking. Unattached sample was removed by washing and a 1:10 dilution of patients sera, containing Brazil specific IgE, added to each peptide coated well. Plates were incubated a further one hour before removing sera by three washes in PBST. Any IgE bound to the peptides was indirectly detected using sequential one hour incubations with monoclonal anti-human IgE (Clone GE-1; Sigma) followed by a rabbit anti-mouse IgG conjugated with peroxidase (Dako) both diluted 1:1000 in PBST. After final washing, enzyme activity was detected by adding 3,3',5,5' tetramethylbenzidine (TMB) substrate (Sigma; T-0440) and the reaction stopped by addition of 100 mM HCl. Absorbance values were determined at both 450 nm and using a 620 nm reference filter (Multiskan EX).

Dot-blotting studies were performed by pre-coating PVDF membrane (Millipore) with 1 µg/ml of recombinant streptavidin (Sigma; S-0677). Membranes were then blocked for one hour with 3% fish gelatine/0.1% Tween 20. Individual peptides were vacuum spotted in microtitre plate format using a template (Schleicher and Schuell) and the membrane coated, then incubated on a rocker overnight with patients' serum diluted 1:40. Membranes were washed in Tris buffer, 0.1% (w/v) Tween, and IgE bound identified as described for ELISA with an alkaline phosphatase conjugated rabbit anti-mouse substituted for peroxidase. Dot-blots were finally developed in BCIP/NBT substrate (Sigma; B-6404) before being dried and scanned.

Preparation of microarray slides

Aldehyde-coated microarray slides (Genetix) were used to bind proteins covalently to the glass surface *via* Schiff base aldehyde-amine chemistry. The purified proteins were diluted to final concentrations of 0.01, 0.03, 0.1, 0.3, 1 mg/ml in PBS (pH 7.4) and spotted using the Biorobotics microgrid II (Apogent Discoveries, USA) to a final spot density of 1920 spots/slide. Each protein dilution is represented by five dots in each slide. The coupling was carried out by incubation for a minimum of four hours in a constant humidity environment. Deactivation of the slides was achieved by two two-minute washes with 0.2% (w/v) SDS, two two-minute washes with water, one five-minute wash with 0.25% (w/v) sodium borohydride in 25% (v/v) ethanol/PBS, three one-minute washes with 0.2% SDS, two two-minute washes with water and finally the slides were dried by centrifugation at 100g for five minutes. All de-activation steps were carried out at room temperature.

Microarray hybridisation

Before use the slides were blocked by incubation with 3% (w/v) BSA in PBST for two hours at 37 °C with shaking. Blood sera were diluted 1:5 and 1:10 (v/v) in PBST to a final volume of 60 µl. The diluted sera were then

transferred to the surface of the slides as six to seven drops and covered by a glass cover-slip avoiding air bubbles. The slides were incubated in a hybridisation chamber for two hours. After the primary incubation the non-reactive antibodies were removed by three two-minute washes with PBST on a slide rack. Specific human IgE was detected by incubation with goat biotinylated anti-human IgE (Vector lab. Inc, Burlingame, USA) diluted 1:2000 (v/v) in PBST for two hours. The slides were then washed for two minutes (three times) with PBST and incubated with streptavidin cy3 (Amersham, UK) diluted 1:2000 (v/v) in 1% BSA, PBST. Unless otherwise stated, all the incubations were performed at 37 °C. The slides were then finally washed for one minute in PBST (ten times), for one minute in water (five times) and dried by centrifugation at 100g for five minutes.

Slide scanning and data handling

An Axon 4000 scanner (Axon Instruments, CA, USA) was used for scanning the dried glass slides with the PMT gain set to 80%. The data were collected with the GenePix software v4 (Axon Inst.) and handled by Microsoft Excel software. The mean of five measures for each protein dilution was considered positive when the fluorescence signal was higher than the average background plus four standard deviations.

Acknowledgements

This work was sponsored by the UK Food Standards Agency (T07019) at the University of Nottingham. The authors thank Professor Elide Pastorello and Dr Laura Farioli, from Ospedale Maggiore di Milano, and Dr S. H. Arshad from David Hide Asthma and Allergy Centre, UK for kindly making available the sera samples used in this study.

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Edited by I. Wilson

(Received 24 May 2004; received in revised form 29 July 2004; accepted 19 August 2004)