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Reduction and alkylation of peanut allergen isoforms Ara h 2 and Ara h 6; characterization of intermediate- and end products

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ABSTRACT

Conglutins, the major peanut allergens, Ara h 2 and Ara h 6, are highly structured proteins stabilized by multiple disulfide bridges and are stable towards heat-denaturation and digestion. We sought a way to reduce their potent allergenicity in view of the development of immunotherapy for peanut allergy. Isoforms of conglutin were purified, reduced with dithiothreitol and subsequently alkylated with iodoacetamide. The effect of this modification was assessed on protein folding and IgE-binding. We found that all disulfide bridges were reduced and alkylated. As a result, the secondary structure lost α-helix and gained some β-structure content, and the tertiary structure stability was reduced. On a functional level, the modification led to a strongly decreased IgE-binding. Using conditions for limited reduction and alkylation, partially reduced and alkylated proteins were found with rearranged disulfide bridges and, in some cases, intermolecular cross-links were found. Peptide mass finger printing was applied to control progress of the modification reaction and to map novel disulfide bonds. There was no preference for the order in which disulfides were reduced, and disulfide rearrangement occurred in a non-specific way. Only minor differences in kinetics of reduction and alkylation were found between the different conglutin isoforms. We conclude that the peanut conglutins Ara h 2 and Ara h 6 can be chemically modified by reduction and alkylation, such that they substantially unfold and that their allergenic potency decreases.

1. Introduction

Peanut allergens Ara h 2 and Ara h 6 are 2S albumins and members of the conglutin family of seed storage proteins [1]. Ara h 2 and Ara h 6 are isoforms of each other, and have been identified as major peanut allergens in *in vitro*, by solid-phase immunoassays and effector cell-based assays [2–7], and *in vivo*, by skin prick tests in peanut-allergic patients [3]. Furthermore, in a mouse model it was shown that immunotherapy with Ara h 2 and Ara h 6 resolved peanut allergy [8], demonstrating that Ara h 2 and Ara h 6 together represent the most relevant peanut allergens in terms of immunotherapy. Ara h 2 and Ara h 6 contain multiple disulfide-bridged cysteine residues, resulting in at least four helical structures that are tightly coiled, heat- and protease-stable [9]. It has been suggested that the tightly coiled protein core may be important for allergenicity of peanut proteins [3,10–12]. Other peanut allergens described in the past, Ara h 1 and Ara h 3, have been shown to be less potent *in vitro*, by effector cell-based assays and *in vivo*, by skin prick tests in peanut-allergic patients [13,14]. Together, Ara h 2 and Ara h 6 represent 80–90% of the allergenic potential of the peanut and are now considered as the most relevant peanut proteins [6,15].

Ara h 2 has two isoforms (Ara h 2.01 and Ara h 2.02), with molecular masses of around 17 and 19 kDa, respectively, coded by homologous genes. The Ara h 2.02 has insertion of 12 amino acids in the middle of the sequence that contains linear IgE-binding epitope [8,16–18]. The mass difference between these two isoforms is 1413 Da [16]. Determination of site-specific proline hydroxylation, disulfide linkages and C-terminal variation of Ara h 2 has been reported previously [19]. It has been shown that Ara h 2 undergoes C-terminal proteolytic processing by endogenous peanut protease [20], resulting in the occurrence of heavy and light isoform of Ara h 2 lacking the C-terminal dipeptide RY. Ara h 6 has been shown to be a potent allergen, sharing epitopes with Ara h 2, with 59% amino acid overall homology and 75% homology in the α-helical regions, resulting in the high degree of immunological cross-reactivity between these allergens (Fig. 1.) [3,21]. Ara h 6 may undergo posttranslational proteolytic processing that involves removal of a dipeptide (IR) from the middle part of the sequence [22], resulting in two polypeptide chains held together by disulfide bonds. Because Ara h 2.02 isoform contains an extra copy of

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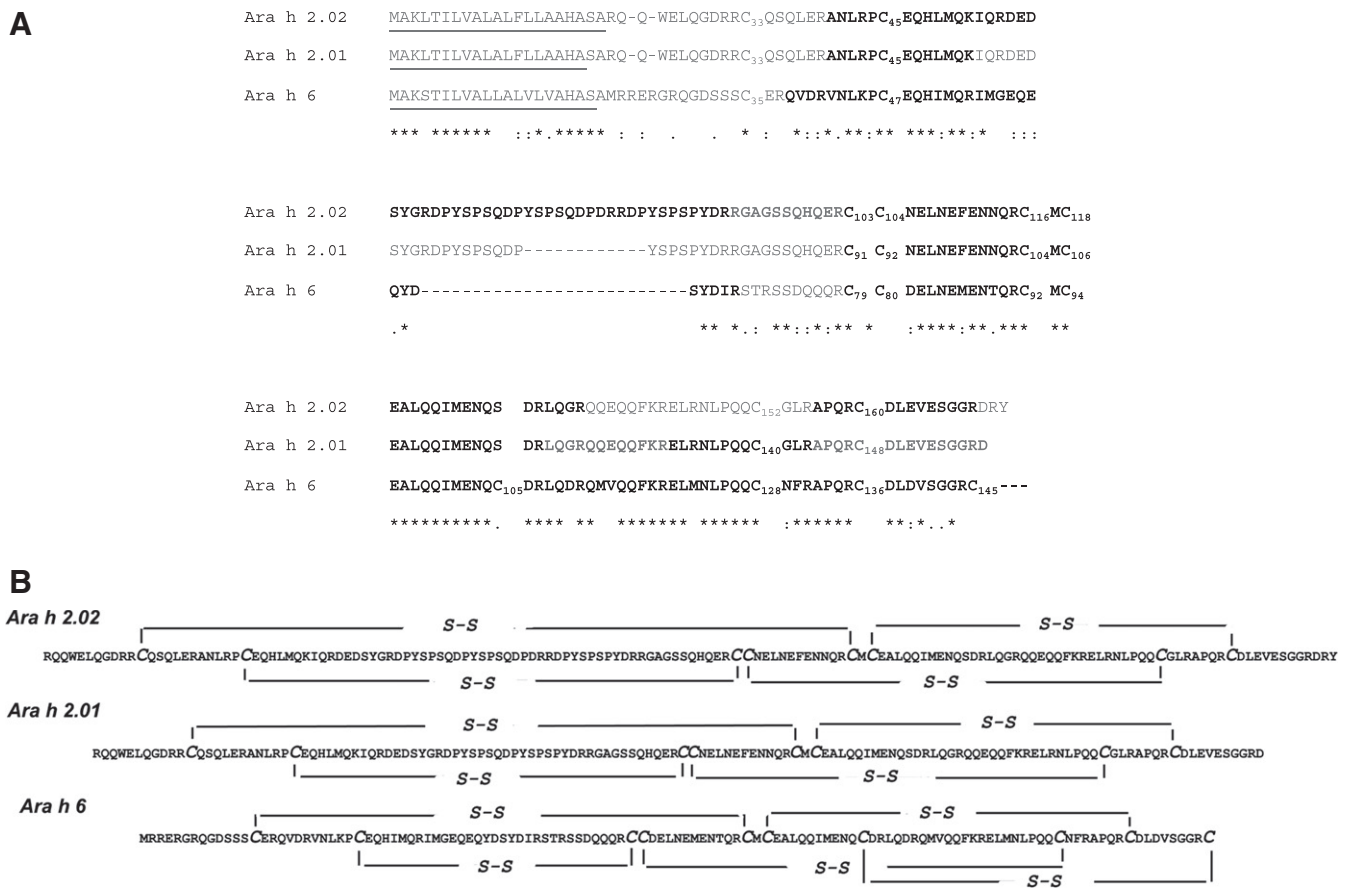

Fig. 1. Primary structure of peanut conglutins. Panel A. Sequence alignment of peanut conglutin isoforms. The following protein identifiers were used in the web-based program ProtParam (www.expasy.org/protparam/), and the protein sequences were taken from www.uniprot.org: Q6PSU2-1, Q6PSU2-4 and Q647G9. Dark letter area: covered sequences by peptide mass finger printing. Panel B. Scheme for disulfides in native conglutin isoforms.
Figure based on the information taken from Li et al. [19] for Ara h 2 isoforms, and from Lehmann et al. [10] for Ara h 6.

an immunodominant epitope, DPYSPS, it has been speculated that this isoform could be more potent than Ara h 2.01 [23,24]. This is supported by Hales et al. [25], who showed that IgE-binding to Ara h 2.02 is more pronounced than for Ara h 2.01. Also, Ara h 2.02 is more efficient in an IgE competition assay [25]. However, Chen et al. [26] reported that Ara h 2.01 has slightly greater allergenic potency than Ara h 2.02, and that repeating linear sequences does not contribute to the allergenic activity of Ara h 2. These differences may be explained by differences in the serology of peanut allergic patients included in these studies. Taken together, peanut derived allergens Ara h 2 and Ara h 6 represent a diverse group of different isoforms of peanut conglutin, the most important allergens in peanut, whose allergenicity is not completely understood.

Within the group of isoforms of peanut conglutin, it has been shown that reduction of the disulfides and subsequent alkylation of the resulting sulfhydryl groups leads to diminished IgE-binding [11,27]. Apparently, the IgE-binding is mainly depending on the protein structure for these allergens. The reduced and alkylated molecules are hypo-allergenic but still immunogenic [12] and potentially suitable for immunotherapy in peanut-allergic patients. The aim of this study is to investigate the critical steps in the reduction and alkylation of peanut allergens to enable the development of consistently modified peanut conglutins.

2. Materials and methods

2.1. Purification of conglutin isoforms

The mix of conglutin isoforms Ara h 2.01, Ara h 2.02, and Ara h 6 was purified as described earlier [3]. To the mix of conglutin isoforms 4 M ammonium sulfate in 20 mM Tris/HCl pH 8 was added to achieve a final concentration of 1.6 M ammonium sulfate. This was applied on a SourcePhenyl 15 column (GE Healthcare, Uppsala, Sweden, 15 mg of protein per 1 ml of column material) previously equilibrated with 1.6 M ammonium sulfate in 20 mM Tris, pH 8.0 (loading buffer). After washing with loading buffer, the column was eluted with a gradient (10 column volumes) of 1.28 M to 0.12 M ammonium sulfate in 20 mM Tris/HCl pH 8. The purity of the collected fractions was tested by SDS-PAGE shown in Fig. 2.

2.2. Kinetics of reduction and alkylation

To assess the adequate concentration of reducing and alkylating reagents, as well as the period necessary for complete modification, kinetic studies were performed. First, 0.5 mg/ml of peanut conglutins in 50 mM sodium phosphate buffer pH 8 were reduced with 0.5 and 5 mM dithiothreitol (DTT). The protein samples were preheated at 60 °C for 0.5 min before reagent addition. Reaction proceeded at 60 °C with continuous shaking, and aliquots (100 μl) were periodically withdrawn (after 0.5, 1, 2, 5, 10, 30, 60 min). The reduction was stopped by immediately adding iodoacetamide (IAA), final concentration of 5 mM and 50 mM, at room temperature in the dark for 90 min. All aliquots were analyzed by SDS-PAGE (non-reducing conditions).

2.3. SDS-PAGE analysis of native and reduced/alkylated conglutin isoforms

Proteins (Ara h 2.02 — heavy isoform, Ara h 2.01 — light isoform, and Ara h 6) were analyzed using a Hoefer Scientific Instrumentation apparatus (Amersham Biosciences, Uppsala, Sweden), on 14% poly-acrylamide gel. Samples were analyzed under both, non-reduced and