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Cysteine-to-serine substitution can alter susceptibility of therapeutic peptides to gastrointestinal enzyme digestion, affecting potential for oral delivery.
ABSTRACT

We recently designed three peptides based on dominant CD4+ T cell epitopes of the major peanut allergen Ara h 2 for inclusion in a peptide-based therapeutic for peanut allergy. In this study, we evaluated and compared the stability of native (cysteine-containing) and therapeutic (serine-containing) forms of these peptides in the presence of different gastrointestinal digestive enzymes, to assess potential for oral administration of these peptides. Two of the three peptides tested were resistant to enzyme digestion at physiological concentrations and therefore show potential for oral delivery. Importantly, significant changes (both beneficial and detrimental) occurred in the resistance of these peptides to high concentrations of enzyme following cysteine to serine substitutions. Our data suggest that such changes could both create and remove enzymatic access to digestion sites, highlighting the complexity of peptide conformation and stability, with implications for therapeutic design.

INTRODUCTION

The development of peptide-based therapeutics for a wide range of conditions has escalated in the last decade, with close to 100 products currently on the market, representing 1.5% of global drug sales. In addition, an estimated 850 peptide-products are now in preclinical or clinical development heralding an even greater expansion of peptide therapeutics in coming years. Peptides hold many advantages over larger molecules such as proteins or antibodies; they are relatively easy to synthesize cost effectively and are amenable to up scaling and standardization. An important advantage of peptides is their amenability to modifications that optimise therapeutic applicability. Considerations for modification include ease of peptide synthesis, solubility, risk of aggregation and stability, and, importantly, their biological reactivity. Cysteine residues are particularly problematic in therapeutic peptides due to susceptibility to oxidation and disulphide bridge formation, which impedes synthesis, solubility, stability and biological reactivity. A frequent approach to avoid these issues is to substitute cysteine residues with structurally conserved, but less chemically reactive, serine residues.

Peptides are being trialled as a new class of therapeutic for allergic diseases, termed SPIRE (Synthetic Peptide Immuno-Regulatory Epitope). Current phase II and III clinical trials show promising results for rapid and long-lasting treatment of allergies to cat, grass pollen and house-dust mites. SPIRE therapy consists of carefully selected short (≤20 amino acids) T cell epitope-based peptides of major allergens, which can ameliorate allergic responses via interactions with T cells, but are too short to cross-link inflammatory cell-bound IgE. This allows for an effective immunotherapy, without risk of the patient experiencing allergic reactions during treatment. These properties make SPIRE therapy an attractive alternative to the current practice of using whole allergen extracts for allergen-specific immunotherapy, particularly for subjects who are unable to use existing products due to the risk of severe reactions during treatment. Furthermore, SPIRE therapy may be the only suitable treatment option for more severe allergies such as peanut allergy, for which no product has yet been approved for specific immunotherapy.

The established route of SPIRE delivery is via intradermal injection, with early trials demonstrating enhanced efficacy compared to subcutaneous administration. Mechanistically, it is thought that intradermal administration enables preferential targeting of peptides to tolerogenic antigen presenting cells (APC), known to be abundant in the dermis. The formation of peptide-HLA complexes on the surface of these cells in the absence of inflammatory signals is thought to facilitate induction of tolerance, deletion and/or regulatory activity in allergen-specific T cells, leading to the observed reduction in allergic response. For this reason, oral delivery could potentially offer an attractive
alternative route of administration. As in the dermis, APC in the mucosa of the oral cavity are thought to promote tolerance induction and efficacy of immunotherapy\(^1\). These tolerogenic APC are not only present in the oral cavity but can also be found throughout the intestinal mucosa\(^2-4\). Finally, in addition to the contribution of tolerogenic immune cells, antigen-specific IgA has been detected in saliva after successful sublingual immunotherapy\(^5\). This type of protection is highly desirable in food allergies. From a practical point of view, oral delivery is less invasive, likely resulting in further improved safety and higher patient compliance. However, for oral immunotherapy to be successful, peptides must first be resistant to degradation by enzymes encountered in the oral cavity and, to some extent, along the digestive tract.

We recently designed peptides based on dominant CD4\(^+\) T cell epitopes of the major peanut allergen Ara h 2 for inclusion in a safe SPIRE therapeutic for peanut allergy\(^6\). We replaced native cysteine residues with serine residues to facilitate production, and demonstrated retained T cell reactivity of the variant peptides\(^6\). Cysteine-to-serine substitutions serve the additional purpose of preventing formation of tertiary structures that could induce inflammatory cell-bound IgE cross-linking and unwanted allergic side-effects. Here we evaluate and compare the stability of native (cysteine-containing) and therapeutic (serine-containing) forms of these peptides in the presence of different gastrointestinal digestive enzymes, to assess potential for oral administration of these peptides in a future treatment for peanut allergy.

**METHODS**

**Peptide specific T cell line generation**

Ara h 2-specific oligoclonal T cell lines (TCL) were expanded from peripheral blood mononuclear cells (PBMC) of peanut allergic subjects using 5,6-carboxyfluorescein diacetatesuccinimidylester (CFSE)-based methodology\(^7\), following a 7 day stimulation with CPE (100\(\mu\)g/mL), Ara h 2 (10\(\mu\)g/mL) or 20-mer peptides (10\(\mu\)g/mL) spanning the Ara h 2 sequence as reported\(^6, 8\). Briefly, TCL specificity was verified by proliferative response to individual Ara h 2 20-mers (10\(\mu\)g/mL) as well as CPE (100\(\mu\)g/mL) and/or Ara h 2 (10\(\mu\)g/mL). Core epitope sequences within the 20-mer peptides were mapped using sets of peptides truncated from the N- or C-terminus of the 20-mer as described\(^6\).

**Enzyme digestion of protein and peptide**

Based on the previously identified dominant epitopes of Ara h 2\(^\dagger\), three peptides, designated peptide 1 (Think Peptides, Clayton, Australia), peptide 2 (Mimotopes, Clayton, Australia) and peptide 3 (Genscript, NJ, USA) were synthesized. Enzymatic digestion of these peptides and crude peanut extract (CPE) was carried out in reaction mixes containing \(\alpha\)-amylase (purified from human saliva), pepsin (from porcine gastric mucosa), trypsin or chymotrypsin (from bovine gastric mucosa) (Sigma-Aldrich, Deisenhofen, Germany) in relevant buffers with pH adjusted as required (Table 1). Mitogenicity and toxicity of the peptides and buffers were tested, as described previously\(^6\). A low and high enzyme:protein ratio were used to mimic biological digestion and to observe the maximum effect of the enzyme, respectively. Equal volumes of enzyme mix were added to protein solution and incubated at 37°C in a water bath. Aliquots were collected at specified time points and heated at 100°C for 5 minutes to stop the digestion reaction. For time point zero, the enzyme was heated prior to addition to the protein solution. Enzymatic activity was evaluated by digestion of CPE following the same protocol and visualization of the resulting fragments with SDS-PAGE. Before use, \(\alpha\)-amylase activity was confirmed using an iodine-starch based assay\(^9\). All enzyme treated samples were aliquoted and stored at \(-80^\circ\)C until further use.

**Table 1. Enzyme digest reaction conditions**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Time points (minutes)</th>
<th>Ratio (enzyme:protein)</th>
<th>pH</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)-amylase</td>
<td>0</td>
<td>3</td>
<td>15</td>
<td>120</td>
</tr>
<tr>
<td>Pepsin</td>
<td>0</td>
<td>5</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0</td>
<td>15</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>0</td>
<td>15</td>
<td>60</td>
<td>120</td>
</tr>
</tbody>
</table>

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

Digested and whole CPE, 15\(\mu\)g/lane, were separated by electrophoresis under reducing conditions using 4% to 12% Bis-Tris SDS-PAGE gels (NuPage, Carlsbad, CA). The gel was stained with Coomassie brilliant blue. Pre-stained standard (1× SeeBlue Plus\(_2\), Invitrogen, Carlsbad, CA) was used as the molecular weight marker.

**T cell proliferation assays**

T cell proliferation assays were performed in duplicate with 10,000 irradiated (5,000 rads) Epstein Barr-transformed B cells and 10,000 peptide-specific TCL incubated with Ara h 2 peptides (10\(\mu\)g/mL) or CPE (100\(\mu\)g/mL) exposed to the digestive enzymes. The
enzymes in the corresponding buffers alone served as negative controls, with untreated peptides in the same buffers as positive controls. These control samples were also heated at 100°C for 5 minutes, in keeping with the protocol applied to the test samples. After 48-hour incubation, 3H-thymidine (0.5μCi/well) was added for the last 16 hours of culture. Thymidine uptake was measured as mean counts per minute (cpm) of replicate cultures. A stimulation index (SI; cpm antigen-stimulated T cells/cpm unstimulated T cells) ≥2.5 was considered positive, indicating adequate peptide presentation for specific T cell proliferation.

Clonality testing of TCL

Clonality of each of the reactive TCL was assessed to exclude the possibility of multiple epitopes being recognized within CPE. TCL were stained with proliferation dye eFluor 670 (eBioscience, CA, USA) according to manufacturer’s instructions and stimulated with peptide or CPE as described in the T cell proliferation assay section. The T cell receptor Vβ repertoire of the reactive TCL was determined using the IOTest BetaMark TCR Vβ Repertoire Kit, as per manufacturer’s instructions (Beckman Coulter, Krefeld, Germany), with the addition of anti-CD4-APC eF750 antibody (eBioscience) for CD4+ T cell identification and 7AAD (BD Pharmingen, Hamburg, Germany) for the exclusion of dead cells.

Enzyme cutting predictors

Predicted sites of digestion by gastrointestinal enzymes were obtained from ExPASy PeptideCutter algorithms (http://www.expasy.org/). Predicted digestion sites did not differ between cysteine and serine versions of the peptides.

RESULTS AND DISCUSSION

We previously identified three short peptides (designated peptides 1, 2 and 3) as candidates for T cell targeted immunotherapy, containing a total of five dominant T cell epitopes of the major peanut allergen Ara h 2 (Figure 1)\textsuperscript{17}. Here we evaluated the proliferative response of T cells to three of these epitopes within the candidate peptides, before and after exposure to several concentrations and durations of enzymatic digestion with α-amylase, pepsin, chymotrypsin and trypsin to represent the main enzymes encountered following oral delivery. Although α-amylase hydrolyses alpha bonds of large polysaccharides and does not digest proteins, it was included to account for any unforeseen effects the enzyme may have on the peptide, interfering with subsequent T cell recognition.

![Peptide 1](http://www.example.org/peptide1.png)

<table>
<thead>
<tr>
<th>Peptide 1</th>
<th>SOLERANLRPCQHLM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 LTLVALALFLAAHASARQQWELQGDRCCS</td>
<td></td>
</tr>
</tbody>
</table>

![Peptide 2](http://www.example.org/peptide2.png)

<table>
<thead>
<tr>
<th>Peptide 2</th>
<th>ELNEFNQVRCMICALQQ</th>
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<tr>
<td>61 PYSPSQPDPSPSPYDRRGAGSSQHQERCCN</td>
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</table>

![Peptide 3](http://www.example.org/peptide3.png)

<table>
<thead>
<tr>
<th>Peptide 3</th>
<th>PQRCDDLVESGGRDY</th>
</tr>
</thead>
<tbody>
<tr>
<td>121 QQEQQFK RELRNLPPQCGRA</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Dominant CD4+ T cell epitopes of Ara h 2.

Five previously identified dominant CD4+ T cell epitopes of the major peanut allergen Ara h 2 are indicated in bold or underlined (when overlapping) within the entire Ara h 2 protein sequence (gray). Peptide 1: Ara h 2(32-47), Peptide 2: Ara h 2(91-108), Peptide 3: Ara h 2(128-141).

Retained T cell proliferative reactivity of each peptide following enzymatic digestion demonstrated that both native and therapeutic forms of all three peptides were resistant to α-amylase (at all concentrations) and pepsin digestion (at physiological concentrations) (e.g. Figure 2a for native peptide 3). In addition, both forms of peptides 1 and 2 were also resistant to chymotrypsin and trypsin digestion at physiological concentrations, indicating that these two peptides, in therapeutic form, are potential candidates for sublingual/oral administration. However, the T cell proliferative reactivity of the native form of peptide 3 was lost within minutes of digestion with low (physiological) concentrations of chymotrypsin or trypsin, demonstrating that peptide 3 was highly susceptible to digestion by these enzymes (Figure 2b). Similar results were obtained for digestion of the therapeutic version of peptide 3 with chymotrypsin (data not shown).
CHAPTER 6

THERAPEUTIC PEPTIDE SUSCEPTIBILITY TO ENZYMATIC DIGESTION

Figure 2. Digestion of peptide 3 by amylase, pepsin, chymotrypsin and trypsin
Proliferative responses of TCL 5 to enzyme alone (enzyme), peptide 3 containing cysteine residues before (peptide) and after 5 time points (t=0 – t=4) of digestion with A. α-amylase (1:300 enzyme:protein ratio, black bars) and pepsin (1:50 enzyme:protein ratio, grey bars) and B. chymotrypsin (1:100 enzyme:protein ratio, black bars) and trypsin (1:500 enzyme:protein ratio, grey bars); C. Peptide 3 sequence with predicted digestion sites from the PeptideCutter program, indicated by vertical lines for pepsin (black dash), chymotrypsin (grey dash) and trypsin (grey solid). Precise TCL epitope indicated below sequence. Cysteine residues indicated in bold were replaced with serine in the therapeutic version of the peptide. n.d: not determined.

The T cell proliferative reactivity to both native and therapeutic forms of peptides 1 and 2 was maintained after enzymatic digestion with trypsin at all concentrations and times tested (data not shown). Upon digestion of the native form of peptide 1 with high concentrations of pepsin or chymotrypsin, T cell proliferative reactivity to the NLRPCEQHL epitope was maintained (TCL 1, Figure 3a). However, T cells specific for a slightly longer version of the epitope (NLRPCEQHLM) no longer responded (TCL 2 and 3, Figure 3a). This indicates a site of digestion between L and M amino acid near the C-terminus of the peptide, consistent with predicted digestion sites for both pepsin and chymotrypsin using peptide cutter programs (Figure 3b). When the same peptide-specific T cells (TCL 2 and 3) were stimulated with CPE after digestion with the same enzymes, proliferation was either entirely (TLC 2) or partially (TCL 3) maintained (Figure 3c).

Although the TCR Vβ specificity of TCL 2 could not be determined with the IOTest Beta Mark TCR Vβ Repertoire Kit (which covers 70% of the human TCR Vβ repertoire), 85% of T cells proliferated in response to peptide 1. Furthermore, clonality of reactive T cells within TCL 3 was confirmed, therefore the possibility of peptides other than peptide 1 being recognized within CPE was minimal (data not shown). These findings demonstrate that the epitope was protected, to some degree, from enzymatic digestion when located within intact allergen. This is in agreement with previous studies evaluating the stability of major peanut proteins, in which Ara h 2 was found to be more stable and resistant to enzymatic digestion with pepsin, chymotrypsin and trypsin than Ara h 1 and 3. The resistance was attributed to the presence of disulfide bonds within the protein, as reduction with dithiothreitol (DTT) prior to digestion resulted in increased fragmentation of the protein21-23. Interestingly, the serine-substituted version of peptide 1 showed a

Figure 3. Digestion of peptide 1 by pepsin and chymotrypsin
A. Proliferative responses of TCL 1, 2 and 3 to enzyme alone (enzyme), peptide 1 containing cysteine or serine residues before (peptide), and after five time points (t=0 – t=4) of digestion with pepsin (1:5 enzyme:protein ratio, black bars) or chymotrypsin (1:100 enzyme:protein ratio, grey bars). B. Peptide 1 sequence, with predicted digestion sites from the PeptideCutter program indicated by dotted lines for both pepsin (black line) and chymotrypsin (grey line). Precise TCL epitopes indicated below sequence. Cysteine residues indicated in bold were replaced with serine in the therapeutic version of the peptide. C. Proliferative responses of TCL 2 and 3 to enzyme alone (enzyme), CPE before (CPE) and after five time points (t=0 – t=4) of digestion with pepsin (1:5 enzyme:protein ratio, black bars) and chymotrypsin (1:100 enzyme:protein ratio, grey bars).
higher resistance to enzymatic digestion by pepsin and chymotrypsin, with no or little reduction in proliferation of TCL 2 and 3. The increased resistance was not predicted by peptide cutter programs suggesting the sequence change could be conferring resistance through changes other than direct alteration of the digestion site.

Assessment of T cell responses to peptide 2 revealed the opposite effect, with retained T cell proliferation to the native cysteine-containing peptide following pepsin and chymotrypsin digestion, but loss of proliferation following digestion of the serine-substituted version by both enzymes (Figure 4a). In this instance, the serine substitution directly affected a predicted chymotrypsin digestion site, but not the predicted pepsin sites (Figure 4b). In contrast to peptides 1 and 2, peptide 3 was resistant to high concentrations of pepsin (Figure 2a) in both the native and therapeutic forms (serine data not shown).

Previous reports on gastrointestinal enzyme digestion of peanut allergens have shown a strong resistance of native Ara h 2 to digestion with trypsin, resulting in a stable ~10 kDa fragment. Sen et al. indicated that this fragment was the result of protective disulphide bridge formation, and that reduction of these bonds prior to digestion inhibited the production of any significant enzyme-resistant fragments. Our results show that epitopes within peptides 1 and 2, both present in the 10 kDa digestion resistant fragment, were still recognized by T cells after trypsin digestion. In contrast, peptide 3 was no longer recognized, indicating its susceptibility to digestion. Sensitization to peanut allergens through oral ingestion would require persistence of digestion-resistant fragments long enough to elicit a response, as is seen following digestion of Ara h 2 and 6. However, IgE epitopes and dominant T cell epitopes can still be found in fragments of other major peanut allergens such as Ara h 1. The demonstration that peptide 1 was protected from digestion with chymotrypsin and peptide when present within CPE, but not as an isolated peptide (Figure 3c), also suggests that allergens such as Ara h 1 may be better protected when ingested within whole peanut than when directly exposed to enzymes as a purified protein in vitro. Alternatively, it could be that sensitization has occurred to these allergens through alternate routes of exposure such as the skin, where the allergens are more likely to remain intact.

In summary, we have demonstrated that peptides derived from dominant T cell epitopes of the major peanut allergen Ara h 2 can resist enzymatic digestion (even at very high concentrations) and therefore have potential to be administered via the sublingual/oral delivery route for treatment of peanut allergy. Importantly, however, we also report significant changes in the resistance of these peptides to pepsin digestion following cysteine to serine substitutions. Enzyme digestion site predictions suggest that changes in enzyme resistance may not only result from removal of digestion sites, but could also be caused by other conformational changes to the peptide. Given the tendency of cysteines to form disulphide bridges, it is quite likely that substitution with serine residues results in significant conformational changes, which directly affect enzyme access to restriction sites. Our data suggest that such changes could both create and remove enzymatic access to digestion sites. This finding highlights the complexity of peptide conformation and stability and emphasizes the need for careful consideration of multiple factors in the modification of peptides for therapeutic use. The data also highlight a need for further research into the implications of using different peptide delivery routes.

![Figure 4. Digestion of peptide 2 by pepsin and chymotrypsin](image-url)

A. Proliferative responses of TCL 4 to enzyme alone (enzyme), peptide 2 containing cysteine or serine residues before (peptide) and after five time points (t=0 – t=4) of digestion with pepsin (1:5 enzyme:protein ratio, black bars) or chymotrypsin (1:100 enzyme:protein ratio, grey bars). B. Peptide 2 sequence with predicted digestion sites from the PeptideCutter program, indicated by dotted lines for both pepsin (black lines) and chymotrypsin (grey lines). Precise TCL epitope indicated below sequence. Cysteine residues indicated in bold were replaced with serine in the therapeutic version of the peptide.
REFERENCES


