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How can thermal processing modify the antigenicity of proteins?

This paper is a brief review of thermally induced covalent modifications to proteins in foods, focussing mainly on the advanced glycation end-products (AGE) of the Maillard reaction. Most foods are subjected to thermal processing, either in the home or during their production/manufacture. Thermal processing provides many beneficial effects, but also brings about major changes in allergenicity. Far from being a general way to decrease allergenic risk, thermal processing is as likely to increase allergenicity as to reduce it, through the introduction of neoantigens. These changes are highly complex and not easily predictable, but there are a number of major chemical pathways that lead to distinct patterns of modification. Perhaps the most important of these is through the reaction of protein amino groups with sugars, leading to an impressive cocktail of AGE-modified protein derivatives. These are antigenic and many of the important neoantigens found in cooked or stored foods are probably such Maillard reaction products. A deeper understanding of thermally induced chemical changes is essential for more advanced risk assessments, more effective QC protocols, production of more relevant diagnostic allergen extracts and the development of novel protein engineering and therapeutic approaches to minimise allergenic risk.

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The wish to make foods safer for victims of food allergy has stimulated numerous attempts to modify ingredients in such a way as to reduce or eliminate their allergenic potential. Yet, despite our ever increasing understanding of allergens and their interactions with the immune system, there still seem to be few, if any, practical ways to achieve this desirable goal.

It is often thought that thermal processing should decrease allergenicity (1), since heating or cooking normally causes a catastrophic disruption of protein structure. Yet the first properly reported case of food allergy (2) was an example of the exact opposite – a food allergy in which the patient was allergic to cooked fish protein, but not to raw fish protein. This is a neat irony for, since that time, there have been few reported cases of food allergy restricted to cooked foods. But there should be no surprise in the often repeated finding that heat treatment does not do much to reduce allergenic risk. There are many ways in which the antigenicity of proteins can be enhanced during thermal processing, especially when this processing takes place in the complex milieu of a food, with so many other ingredients available to participate in complex physical and chemical reactions (3).

Thermal processing, potential neoantigens and allergenicity

Thermal processing is a necessary and unavoidable complication to the problem of assessing the potential allergenicity of foods, food ingredients and processes. Any kind of processing, even storage, is likely to introduce new allergenic potential, yet most foods must be cooked for safety reasons, as well as to provide the flavours, textures and processing qualities needed for palatability and stability (4). So, the challenge for food scientists, toxicologists, food manufacturers and clinical allergists is to better understand the effects of (thermal) food processing on allergenicity, and then take actions to minimise the impact on allergic consumers.

Improved understanding begins with an appreciation that plentiful, stable, neoantigens are always formed when foods are cooked (or stored in the presence of oxygen), even though other, usually conformational, epitopes are removed at the same time. In some cases, the net effect is to increase allergenicity, while in others, heating decreases allergenicity or leaves it unchanged. There are no absolute rules. Cooking can eliminate allergenicity, as in the work of Uriso et al. (5), with patients who were allergic to freeze-dried egg white, but who did not react to cooked egg white.

Because of this complexity, this paper is focussed mainly on process-dependent covalent modifications that modify *antigenicity* (3), rather than attempting to cover the full spectrum of changes in digestibility, solubility, resistance to stomach acid and the ability to be absorbed intact across the gut mucosa. These are all essential factors that must be considered when exploring the full impact of processing on overall *allergenicity*. In judging the allergenic risk of neoantigens, it would also be necessary to consider the context in which the modified

allergen is presented to the gut. The digestion conditions affecting a protein ingested as part of a meal or a multi-component food are different to those experienced by the same protein ingested in isolation.

Maillard products and advanced glycation end products (AGEs) as neoantigens

Perhaps the most important and well-defined covalent modification of proteins is that which occurs through the Maillard reaction, resulting in the attachment of reducing sugars and sugar breakdown products, mainly to free β-amino groups of lysines (Fig. 1). It is now clear that even sucrose, a non-reducing sugar very widely used in processed foods, can participate in these reactions, for we (CMS and DCJ) have shown that sucrose readily hydrolyses at the temperatures reached during normal processing, thus releasing glucose and fructose to modify the proteins present (6). The reactions take place rapidly at or above 100°, when the sugar anomeric carbon reacts with the nitrogen of available amino groups, to form a Schiff's base. But, to understand the full impact of this chemistry, it must be appreciated that the reaction sequence doesn't stop there. The Schiff's base undergoes an Amadori rearrangement to yield the corresponding ketoamine (7). Fructose reacts more weakly, but in a broadly similar way; the subsequent rearrangement follows a slightly different path, to form the Heyns product (8). Although these relatively stable reaction products (especially the Amadori product) accumulate as prominent components of the processed food, by the end of the process they are not alone. Glycation is just the first step of a poorly understood complex reaction cascade (Fig. 2), resulting in the formation of variable amounts of numerous protein adducts that give the brown coloration often desired in cooked foods. The same reactions go on at a slow rate during storage, and they also occur *in vivo*, especially in individuals with higher than normal levels of glucose (as in poorly controlled diabetes), where the modifications can cause pathological changes through glycation of key functional or structural proteins. These products are known collectively as advanced glycation end products (AGEs) and have been observed after antiviral thermal bioprocessing in model protein formulations, which mimic those found in therapeutic protein preparations (6). One of the main reaction products of this process is known as carboxymethyl lysine (CML), formed by reaction with dicarbonyl intermediates of sugar breakdown, as shown in Fig. 1 (6).

Other covalent modifications of proteins caused by heating or storage can contribute to changes in antigenicity. These include reactions with oxidised lipids (9), direct oxidation through reactive oxygen intermediates (10), disulphide bond scrambling and deamination of asparagine (3). Reactions with polyphenols in many plant-derived foods can cause substantial and unpredictable changes in protein structure.

But, when considering the safety implications of thermal processing, the main questions are whether:

- these and similar chemical modifications, acquired through thermal processing or storage are recognised by the immune system as strong B-cell epitopes;
- the whole modified antigens (or a sufficiently large, conserved part) are sufficiently stable to be absorbed intact;
- the absorbed modified antigens can evoke a Th2 response in such a way as to generate IgE anti-bodies to the neoepitopes;
- the modification occurs in a sufficiently reproducible way to provoke allergic **sensitisation** and subsequent reaction.

AGE neoantigens and food allergy

AGE neoantigens are important B-cell epitopes, for there are many examples of clinically significant antibodies against AGE-modified proteins, such as those naturally formed *in vivo* on tissue proteins (11) or on therapeutic preparations of factor VIII after thermal processing (12). Ikeda et al. characterized a group of antibodies to CML, and also identified two other groups of AGE antigenic structures (13, 14). However, in the field of food allergy it must be unlikely that CML itself could constitute a complete epitope for an IgE antibody, for the consequence would be an allergy to virtually all cooked foods, regardless of their origin and protein composition. Such nonspecific, wide ranging food allergies are not observed, so it must be assumed that CML and other types of AGE B-cell epitopes also include unique parts of the protein on which they are carried.

The ability of such neoantigens to work as full-blown allergens (by being naturally absorbed across the mucosa and provoking IgE responses) would be confirmed by clear case reports of allergy exclusively against AGE modified proteins. In fact, there are some well described, published case studies of allergic reactions to neoantigens present only in processed/stored foods, but they do not provide direct evidence of the particular chemical modifications responsible. Malanin et al. (1) described an anaphylactic reaction to pecan nuts in cookies in an 8-year-old girl. She had IgE antibodies specific for pecan antigens formed during storage or heating, and which were demonstrably absent from fresh pecan nuts. These neoantigens were tracked by means of immunoassays in which the patient's serum was used as tracer reagents, and they appeared abruptly after less than 2 weeks of storage or after roasting. By comparison with other, well characterised systems, it seems likely that these pecan neoantigens resulted from the Maillard reaction. For

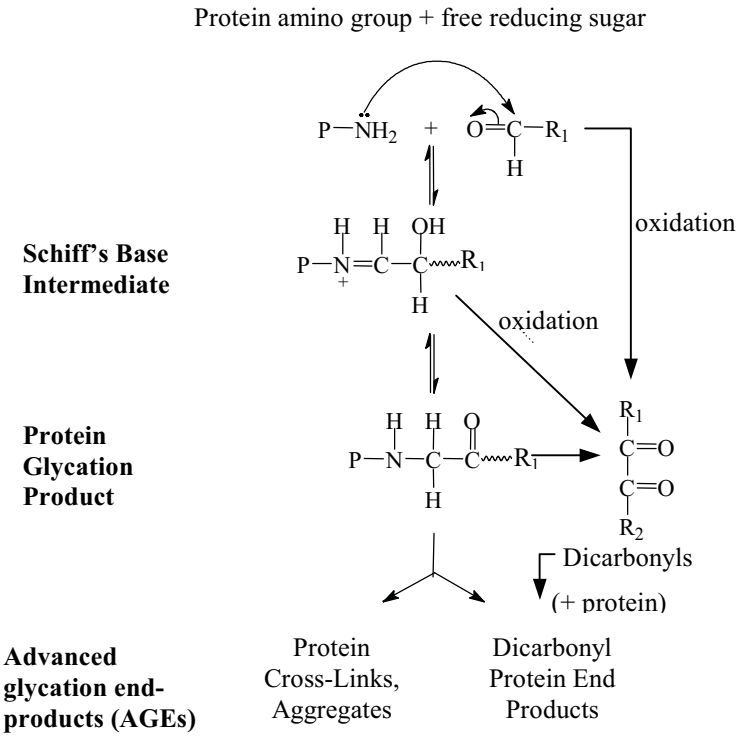

Figure 1. The mechanism for the reaction of protein amino groups with reducing sugars.

example, potent neoantigens also appeared in (uncooked) wheat flour extracts after inappropriate storage for 7 months at ambient temperature (15). In this example there was no reaction when colourless fresh wheat extract was injected into the patient (diagnosed with baker's asthma), but there was a severe anaphylactic reaction when the distinctly brown, stored extract was injected. The problem of thermally derived neoantigens was further highlighted in a study on the adequacy of food extracts for skin testing, when one of the patients was found to have a strong reaction to extract of cooked shrimp, but no reaction to extract of raw shrimp (16).

Although not food ingredients, soybean hulls have provided an important example of the potency of heat-induced neoallergens (17). In an investigation with a panel of 68 soybean asthmatic subjects, fresh soybean hulls were only weakly allergenic but, after heating under storage and transportation conditions, they contained potent neoallergens, which appeared to be responsible for the soybean-induced asthma.

The scarcity of such examples, in which the allergy is restricted only to processed foods, might give the impression that neoantigens are not generally important in food allergy. However, their real significance has more to do with substantially enhanced allergenic potency, rather than with an all-or-none effect. For example, it was reported a long time ago (18) that β-lactoglobulin heated at 50°C in the presence of lactose acquired a 100- fold increase in skin reactivity. Some of this was apparently due to direct complement activation by the covalently attached sugar groups but, in addition, there was a major increase in binding of IgE antibodies, presumably to the new B cell epitopes created (19). When taken with evidence on the effects of AGE-modified proteins in a wide range of other clinical conditions (11), these results suggest that thermally generated glycation products must also be important in food allergy. Even so, there have been surprisingly few rigorous studies on the role of AGEs in food allergy.

In a very recent study on the effects of roasting on the allergenic properties of peanut proteins, Maleki et al. (20) clearly demonstrated, perhaps for the first time, the particular effect of the Maillard reaction on the allergenicity of purified individual proteins (Ara h 1 and Ara h 2). To be certain that the Maillard modifications were, themselves, responsible for at least some of the allergenicity changes, a well-defined experimental system was used. It was found that Maillard modifications of Ara h 1 and Ara h 2 were, indeed, responsible for substantial increases in the allergenicity of the purified proteins (as determined by the binding of IgE antibodies from allergic patients). But, as expected, these particular covalent modifications were only a part of the 90-fold increase in the allergenicity of peanuts after roasting. In addition, the Maillard-modified proteins were found to be markedly more resistant to digestion than native proteins, thus enhancing their allergenic potential.

Conclusions

The modification of protein antigenicity in foods by thermal processing is complex, especially as there are so

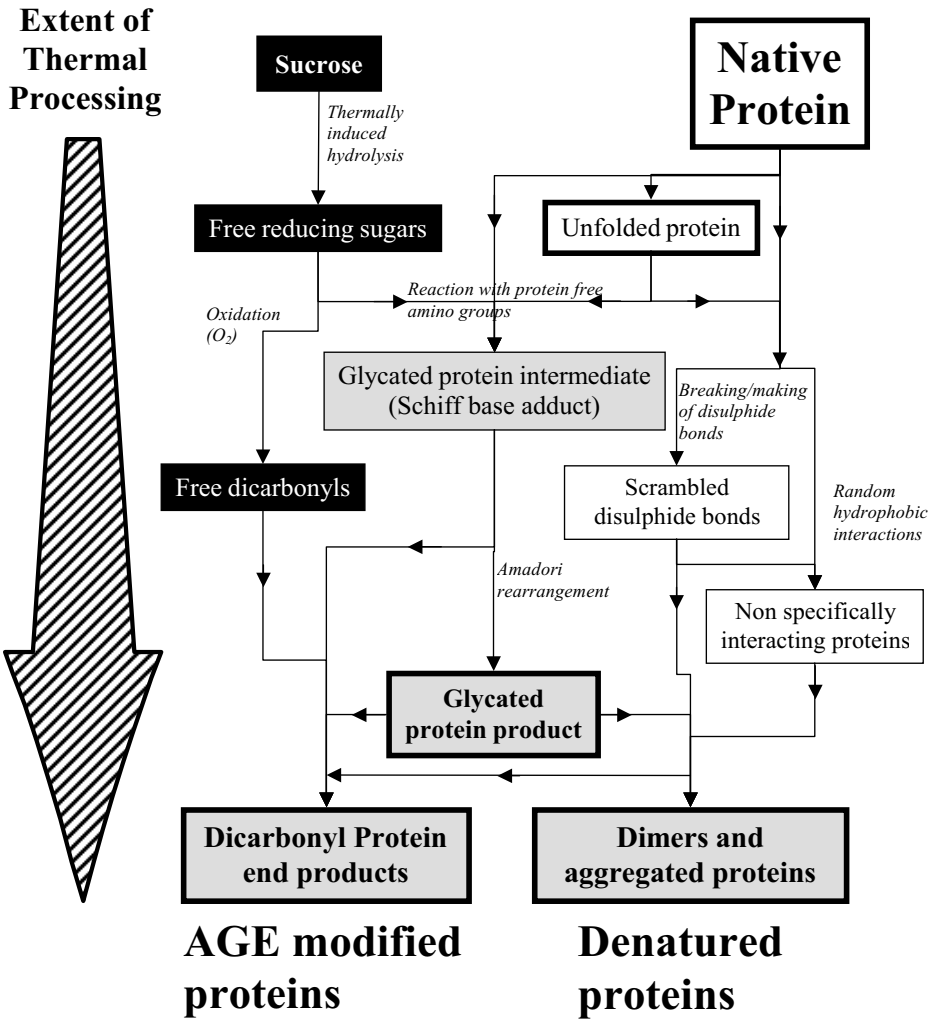

Figure 2. A diagrammatic overview of the chemical modifications to proteins caused by thermal processing in the presence of sucrose. Non-protein components are shown as black boxes, proteins without covalent substitutions as white boxes and covalently substituted proteins as grey boxes. Proteins that are present at the end of the process potentially bearing neo-epitopes are in boxes enclosed by a bold line.

many variables within the composition, processing conditions, circumstances of exposure to the consumer and the particular responsiveness and genetic make-up of the individual. But AGE modifications are formed through an important chemical pathway, leading to distinct, if complex, patterns of protein derivatives. These are likely to be key sources of thermally induced neoantigens.

A more detailed understanding of such general routes of covalent protein modification can make foods safer, if it is used to:

- make patients, clinicians and dieticians more aware of the need to consider not just the identity/source of ingredients but also the processing which they have received;
- allow more informed and accurate risk-analysis of new ingredients, novel proteins and new processes;
- enable more effective approaches to minimising allergic risk through protein engineering, novel therapies, tolerogenic vaccines, etc.;
- provide a basis for more relevant, better controlled food extracts for use in diagnosis (*in vivo* and *in vitro*).

In our relentless pursuit of food safety, we need still more understanding of the allergenicity-modifying chemical reactions that take place during all kinds of processing, despite their daunting complexity. There is an enormous need and opportunity for fundamental and applied research on this topic.

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