



Relationship between protein digestibility and allergenicity: Comparisons of pepsin and cathepsin

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ABSTRACT

An association between protein allergenicity and resistance to pepsin digestion in the gastrointestinal tract has been proposed. However, although widely accepted, such an association is inconsistent with known labile allergens and resistant nonallergens. Given the central role of antigen presenting cells, and in particular dendritic cells (DC), in the development of allergic responses, the stability of allergens to intracellular processing may be more relevant than resistance to extracellular pepsin digestion. We have characterised the expression by DC of cathepsins (proteolytic enzymes), and compared the proteolytic activity of the most highly expressed cathepsin with pepsin for a range of 9 allergens and 4 putative nonallergens. Cathepsin expression in bone marrow-derived DC (BM-DC) derived from BALB/c strain mice was characterised by flow cytometry; cathepsins D, E and S were identified, with cathepsin D being the most highly expressed. Digestion studies revealed that the majority of allergens (5/9) were pepsin resistant, whereas non-allergens (3/4) were labile. If the generation of pepsin-resistant fragments was considered as a feature of allergenicity, this increased to 7/9 allergens and 4/4 nonallergens. In contrast, most of the proteins examined were resistant to cathepsin digestion, with significant digestion recorded for only 2/9 allergens and 2/4 non-allergens. Chemical reduction (to mimic intracellular reducing conditions) increased the susceptibility of proteins to digestion by cathepsins, but did not improve discrimination between allergens and nonallergens on this basis. These data confirm that there is a general relationship between resistance to digestion with pepsin and allergenicity. The relationship is not absolute, but the information gained from this characteristic does provide useful information in a weight of evidence approach for allergenicity assessment. The most abundant cathepsin detected in antigen processing BM-DC, cathepsin D, is not an appropriate substitute for pepsin. The hypothesis that pepsin stability may be a surrogate for stability to digestion within DC may still hold true, but consideration of a single enzyme in this context is possibly an oversimplification.

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1. Introduction

The prevalence of IgE-mediated food allergies has been increasing, with 2% of US adults and up to 10% of children (those living in urban areas) estimated to be affected (Gupta et al., 2012; Vickery et al., 2011). Although there are reports of allergic reactions to many foodstuffs, most cases of food allergy are associated with a limited number of foods including peanuts, hens' egg, cows' milk, fish and

shellfish (Robison and Pongracic, 2012). There is good evidence that changing patterns of dietary consumption can result in changes to the profile of the foodstuffs most commonly associated with allergy. Thus, since its introduction into European diets some 40 years ago, kiwifruit is now among the top 10 inducers of food allergy in some European countries (Ballmer-Weber and Hoffman-Sommergruber, 2011; Lucas et al., 2003). Related to this is the fact that an important safety concern regarding the introduction of genetically modified crop plants is the potential for allergenicity (Selgrade et al., 2009).

Currently, safety assessment of genetically modified products requires consideration of various parameters including assessment of homology with known allergens using various *in silico* databases, IgE binding studies and resistance of the protein to digestion with simulated gastric fluid (SGF) (Selgrade et al., 2009). Thus, in a recent study investigating the safety of the protein osmotin, expressed in transgenic crops to enhance abiotic stress tolerance, bioinformatic analyses revealed homology with fruit allergens including tomato and apple. Serological identity with IgE-containing serum samples

Abbreviations: AP, allophycocyanin; APC, antigen presenting cell; AU, arbitrary units; BM, bone marrow; BME, beta-mercaptoethanol; BSA, bovine serum albumin; CM, culture medium; DC, dendritic cell; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; PBS, phosphate buffered saline; RUBISCO, ribulose-1,5-bisphosphate carboxylase oxygenase; PE, phycoerythrin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SGF, simulated gastric fluid.

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from apple and tomato allergic individuals was demonstrated and the protein was also resistant to pepsin digestion under standard conditions (Sharma et al., 2011). As such, osmotin was regarded as being a potential allergen.

The relationship between allergenicity and stability to digestion and the most appropriate experimental conditions for measurement of stability have been the subject of some debate. The initial report by Astwood et al. (1996) demonstrated that many animal and plant food allergens displayed resistance to pepsin digestion, whereas other very common plant proteins, believed not to be allergenic, were digested rapidly (within 30 s). However, in subsequent studies the relationship between resistance to digestion and allergenicity was found not to be absolute (Fu et al., 2002; Lucas et al., 2008; Thomas et al., 2004). Thus, there are examples of relatively labile allergens, including some kiwifruit allergens and the peanut allergen Ara h 2, and stable nonallergens such as the lectin concanavalin A. Furthermore, there has been controversy regarding the most relevant pepsin:protein ratio and the pH of SGF to be utilised. The original publication used a pH of 1.2 (Astwood et al., 1996), but others have argued that a higher pH may be more physiologically relevant. Many proteins exhibit marked sensitivity to pH changes, with both the kiwifruit allergen actinidin and codfish allergens being extremely labile at low pH, but stable to pepsin digestion at above pH 2.5 (Lucas et al., 2008; Untermayr et al., 2005). This has particular relevance for patients taking acid-suppression medications for dyspeptic disorders, such that gastric pH is increased. Such treatments have been shown to increase the risk of food allergy in man and in experimental animals (Riemer et al., 2010).

The potential physiological relevance of the association between protein stability and the development of allergic responses is also of interest. It is clear that those proteins which are pepsin-resistant will persist longer in the gut environment and therefore have greater opportunity to interact with the gut immune system. However, even those proteins that are very rapidly digested with pepsin have been shown to be able to stimulate an immune response following oral administration to mice, indicating sufficient exposure to allow engagement with the immune system (Dearman et al., 2002). This leads to the hypothesis that resistance to extracellular digestion by pepsin may be a surrogate for another property: that of resistance to digestion within the antigen-processing and presenting cells (APC) of the immune system. To initiate an immune response, exogenous protein is broken down under reducing conditions into peptides by enzymes that reside within endosomal and lysosomal compartments of APC, and those peptides are then displayed on the cell surface (Li et al., 2005). Dendritic cells (DC) are “professional” APC, capable of initiating immune and allergic responses, and expressing a number of proteolytic enzymes called cathepsins (Honey and Rudensky, 2003; Hsing and Rudensky, 2005).

In the current experiments, the cathepsin profile of mouse bone marrow derived-DC (BM-DC) has been characterised and studies performed comparing the resistance of various allergens and putative nonallergens to digestion by pepsin and by the most highly expressed BM-DC cathepsin under reducing and non-reducing conditions.

2. Materials and methods

2.1. Animals

Young adult (6–10 weeks old) female BALB/c strain mice (Harlan Olac, Bicester, UK) were used throughout these studies. Mice were housed on sterilised wood bedding with materials provided for environmental enrichment. Food (Beekay Rat and Mouse Diet No1 pellets; B&K Universal, Hull, UK) and water were available *ad libitum*. The ambient temperature was maintained at $21 \pm 2^\circ\text{C}$ and relative humidity was $55 \pm 10\%$ with a 12 h light/dark cycle. All procedures were approved by the UK Home Office and carried out in compliance with the Animals (Scientific Procedures) Act, 1986 under a Home Office granted Project Licence.

2.2. Generation and culture of DC

BM-DC from BALB/c strain mice were generated as described previously (Dearman et al., 2009). The culture medium (CM) used throughout these studies was RPMI 1640 with 10% heat inactivated fetal calf serum (FCS), supplemented with 400 $\mu\text{g}/\text{ml}$ streptomycin, 400 $\mu\text{g}/\text{ml}$ penicillin (all GIBCO, Paisley, Renfrewshire, UK) and 50 μM β -mercaptoethanol (BME, Fisher Scientific, Loughborough, UK). BM cells were flushed from the femurs and tibias with phosphate buffered saline (PBS) and viable cell counts performed by exclusion of 0.5% trypan blue. Cells (2×10^6) were plated out in sterile petri dishes (100 mm $\times$ 15 mm) in 10 ml of CM supplemented with granulocyte/macrophage colony stimulating factor (Peprotech, London, UK; 20 ng/ml) and cultured in a humidified atmosphere of 5% CO_2 at 37°C . The cultures were fed on day 3 by addition of 10 ml of fresh CM supplemented with cytokine, and again on day 6 and 9 by gentle aspiration of 10 ml of medium and addition of 10 ml of fresh CM supplemented with cytokine. At day 12, the loosely adherent clusters were dislodged and harvested gently with sterile pasteur pipettes and resuspended in 5% FCS in PBS for flow cytometry or resuspended (10^6 cells/ml) in fresh CM (supplemented with cytokine), and 1 ml aliquots (final volume) seeded into 24 well tissue culture plates. Cells were either analysed for endocytic activity or cultured in the presence or absence of 100 ng/ml lipopolysaccharide (LPS) from *Escherichia coli* serotype 055:B5 (Sigma Chemical Co., St. Louis, USA) or medium alone for 24 h and processed for flow cytometry.

2.3. Flow cytometric analyses

2.3.1. Phenotypic markers

Approximately 2×10^5 DC/well were incubated in round-bottomed 96 well tissue culture plates for 30 min with monoclonal antibodies directed against I-A^d/I-E^d (2.5 $\mu\text{g}/\text{ml}$; clone 2G9, rat IgG_{2a} κ ; Pharmingen, San Diego, CA), CD40 (10 $\mu\text{g}/\text{ml}$; clone 1C10, rat IgG_{2a} κ ; Chemicon, Chesham, Hampshire, UK), CD54 (10 $\mu\text{g}/\text{ml}$; clone KAT-1, rat IgG_{2a} κ ; Chemicon), CD80 (10 $\mu\text{g}/\text{ml}$; clone IG10, rat IgG_{2a} κ , Pharmingen), CD86 (10 $\mu\text{g}/\text{ml}$; clone GL1, rat IgG_{2a} κ ; Pharmingen), or with purified rat IgG_{2a} κ (10 $\mu\text{g}/\text{ml}$; R&D Systems, Abingdon, UK). This was followed by incubation with goat anti-rat IgG fluorescein isothiocyanate (FITC) (5 $\mu\text{g}/\text{ml}$; Serotec, Oxford, UK) for 30 min. Alternatively, cells were incubated with CD11c-phycoerythrin (PE) or PE-conjugated golden syrian hamster IgG1 κ isotype control (4 $\mu\text{g}/\text{ml}$; Pharmingen) for 30 min. All incubations were performed in 5% FCS in PBS at 4°C . Dead cells were excluded from all analyses by staining with 5 $\mu\text{g}/\text{ml}$ propidium iodide (PI, Sigma) or 0.5 $\mu\text{g}/\text{ml}$ 7-amino actinomycin D (Pharmingen) immediately prior to analysis.

2.3.2. Identification of intracellular cathepsins

Cells ($\sim 2 \times 10^5$ DC) were transferred into round-bottomed 96 well plates and incubated for 10 min with CD16/32 and hamster IgG (both at 5 $\mu\text{g}/\text{ml}$; Pharmingen) to block non-specific binding. Subsequently, cells were incubated for 30 min at 4°C with PE conjugated CD11c antibody or PE conjugated hamster IgG1 κ isotype control (both diluted to 4 $\mu\text{g}/\text{ml}$). Cells were then fixed with 2% paraformaldehyde followed by permeabilisation using 0.1% saponin (both for 10 min at room temperature in the dark and from Sigma). This was followed by incubation for 30 min with 10 $\mu\text{g}/\text{ml}$ affinity purified biotinylated goat anti-mouse cathepsin D or E antibodies (R&D Systems) or 4 $\mu\text{g}/\text{ml}$ rabbit polyclonal cathepsin S antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with their appropriate isotype controls; goat IgG (R&D Systems) for cathepsin D and E or rabbit IgG (Santa Cruz Biotechnology) for cathepsin S. Finally, cells were incubated with goat streptavidin allophycocyanin (AP) conjugate (cathepsins D and E antibody/isotype) or rabbit streptavidin AP conjugate (cathepsin S antibody/isotype) for 30 min at 4°C .

2.3.3. Endocytic activity of BM-DC

Cells were seeded at 1×10^6 cells/well into 24 well plates and incubated for 1 h at either 37°C or 4°C in the presence or absence of 20 mM NaN_3 . Subsequently, 1 mg/ml FITC-bovine serum albumin (FITC-BSA; Sigma) was added to the cells and the cells maintained at either 37°C or 4°C for 30 min. Endocytosis was stopped by washing 5 times in ice-cold 5% FCS in PBS. Dead cells were identified and excluded from analysis using PI.

All samples for flow cytometry were analysed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) and CellQuest Pro software (Becton Dickinson), 10^4 cells were acquired per sample.

2.4. Digestibility of proteins

2.4.1. Test proteins

The source, supplier and nomenclature of the test proteins utilised in these assays are shown in Table 1. Digestibility of native test proteins and chemically reduced test proteins was examined. For chemical reduction, proteins at 20 mg/ml in water were incubated with 2.4 M BME and 8 M urea (Sigma) for 2 h at 37°C , then dialysed extensively against 0.05% acetic acid.

2.4.2. Cathepsin D digestibility

Test proteins (10 mg/ml) were dissolved in 0.1 M NaOAc, 0.2 M NaCl, pH 3.5. Samples were incubated at 37°C overnight alone or in the presence of 30 $\mu\text{g}/\text{ml}$ recombinant mouse cathepsin D (R&D Systems). Further controls were incubated

Table 1
Details of allergens and putative nonallergens.

Allergens	Abbreviation	Source	Supplier	Allergen family	Designation
Actinidin	Act	Kiwifruit	NZP	Papain (cysteine protease)	Act d 1
Ara h 1	NA	Peanut	TNO	Cupin	Ara h 1
Ara h 2	NA	Peanut	TNO	Prolamin	Ara h 2
β-Lactoglobulin	BLG	Bovine milk	Sigma	Lipocalin	Bos d 5
Bovine serum albumin	BSA	Bovine milk	Sigma	Serum albumin	Bos d 6
Bromelain	Brom	Pineapple	Sigma	Papain (cysteine protease)	Ana c 2
Hens' egg lysosome	HEL	Hens' egg	Sigma	Glycoside hydrolase	Gal d 4
Ovalbumin	OVA	Hens' egg	Sigma	Serpin	Gal d 2
Patatin	Pat	Potato	Syngenta	Patatin-like phospholipase	Sol t 1
Soy trypsin inhibitor	STI	Soya bean	Sigma	Kunitz-type STI	Gly m TI
Putative nonallergens					
Concanavalin A	Con A	Jack beans	Sigma	NA	Not in database
Hemoglobin	Hb	Bovine	Sigma	NA	Not in database
Horse radish peroxidase	HRP	Horse radish	Sigma	NA	Not in database
Ribulose-1,5-bisphosphate carboxylase oxygenase	RUBISCO	Spinach	Sigma	RUBISCO	Spi o RuBisCO

The test proteins utilised have been characterised as allergens and nonallergens. Full names, standard abbreviations and supplier details are shown. For allergens, the allergen family and the allergen nomenclature as classified in the database at www.allergome.org are shown. NA, not applicable.

with cathepsin D and 1 μ M pepstatin A (Sigma). The reaction was stopped by neutralisation with NaHCO₃ and heating to >75 °C in 5 $\times$ Laemmli sample buffer (40% glycerol, 5% BME, 10% sodium dodecyl sulfate [SDS; Sigma], 0.33 M Tris, 0.05% bromophenol blue [Bio-Rad Laboratories, Hemel Hempstead, UK], pH 6.8). Samples were analysed immediately by SDS-polyacrylamide gel electrophoresis (PAGE).

2.4.3. Pepsin digestibility

The method used was based upon the multi-laboratory study published by Thomas et al. (2004). Simulated gastric fluid (SGF; 0.084 N HCl, 35 mM NaCl, pH 1.2, and 4000 units of porcine pepsin [Sigma]) (1.58 ml) and 0.08 ml of test protein solution (5 mg/ml) was prepared. After mild vortexing, the samples were incubated at 37 °C in a water bath and 200 μ l aliquots were removed after 0, 0.5, 2, 5, 10, 20, 30 and 60 min and immediately quenched with 70 μ l of 5 $\times$ Laemmli buffer and 70 μ l of 200 mM NaHCO₃ pH 11 and placed in a heat block at >75 °C for approximately 10 min. Controls for pepsin auto-digestion (pepsin without test protein) and controls to show test protein stability under test conditions in the absence of pepsin (reaction buffer with test protein but without pepsin) were included. Aliquots of these control samples were taken at 0 and 60 min only. Samples were stored at –20 °C prior to analysis.

2.4.4. SDS-PAGE

Samples were subjected to SDS-PAGE under reducing conditions, using 15-well, 0.75 mm thick, 10% polyacrylamide peptide gels made in house. Mark10 standard markers (Bio-Rad) containing 10 proteins ranging in size from 10 to 250 kDa were included on all gels. A tricine buffer (100 mM Tris, 100 mM Tricine, 0.1% SDS pH 8.2; Bio-Rad) system was used for resolving small polypeptides. Samples were loaded at 10 μ l/well and markers at 5 μ l/well and run at 125 V. Gels were fixed for less than 5 min in 5% trichloroacetic acid and washed in 45.5% methanol/9% glacial acetic acid for 1 h. The gels were then stained with Coomassie biosafe brilliant blue solution (Biorad, G-250) for 1 h and destained in water. Gels were scanned using a Li-cor gel scanner (Li-cor Biotechnology, Lincoln, NE, USA) and densitometric analysis carried out using Odyssey® software (Li-cor). Integrated intensity (intensity and area of band following subtraction of background values) was calculated to facilitate between gel comparisons. The percentage change in band density compared with the time zero value was calculated. Fragments of proteins produced by digestion with pepsin or cathepsin were not measured by densitometry but their absence or presence was recorded.

2.5. Statistics

The statistical significance of changes in membrane marker expression and uptake of FITC-BSA recorded by DC was analysed by two-way ANOVA and Bonferroni post hoc tests. Significant differences in the expression of cathepsin D, E or S by BM-DC were assessed by one-way ANOVA and Tukey's post hoc test. Changes in band intensity following pepsin or cathepsin digestion were assessed by one-way ANOVA and Dunnett's multiple comparison test.

3. Results

In initial experiments, the maturation status of BM-DC was investigated. Kinetic studies conducted using days 4, 6, 8, 10, 12 and 14 day BM-DC (data not shown) revealed that day 12 DC were relatively pure DC with an immature phenotype and able to endocytose

antigen efficiently. The DC-specific marker CD11c, CD54 (intra-cellular adhesion molecule-1) and the maturation markers major histocompatibility (MHC) class II, CD80, CD86 and CD40 were analysed on day 12 resting DC and DC that had been stimulated for 24 h with 100 ng/ml of the DC activator LPS (Fig. 1). The majority of day 12 resting cells expressed a DC phenotype, with ~80% positive for CD11c, CD54 and MHC class II. Consistent with an immature phenotype, resting DC were only 30% positive for CD80 and CD86 and <10% were CD40 positive, with these markers being expressed at low levels (mean fluorescence intensity [MFI] < 5). Culture with LPS resulted in maturation of the DC population; although CD11c and CD54 were unchanged, the frequency of CD86 and CD40 expression increased significantly. In addition, the levels of expression of CD54, CD86 and CD40 were significantly elevated. The ability of day 12 BM-DC to take up fluorescently labeled protein (FITC-BSA) was examined also. In order to differentiate between surface binding of FITC-BSA and active uptake, cells were incubated at 4 °C and at 37 °C in the presence or absence of the metabolic inhibitor NaN₃ (Fig. 1). There was little effect of either temperature or azide on the percentage of cells positive for FITC-BSA, with approximately 60–70% of cells positive for fluorescence regardless of incubation conditions. However, the uptake per cell was markedly affected by temperature and NaN₃. Relatively low MFI levels of <60 arbitrary units (AU) that were not affected by NaN₃ were recorded at 4 °C, consistent with binding without active uptake. At 37 °C there was active uptake with MFI values approaching 900 AU and significant inhibition in the presence of NaN₃ to <300 AU.

The profile of day 12 BM-DC being consistent with immature DC with marked endocytic activity, and thus antigen-processing capacity, the next step was to characterise the cathepsin profile of these BM-DC. Cells were identified as CD11c positive (DC) and then after permeabilisation were stained for the intracellular expression of a range of cathepsins (cathepsins D, E and S) (Figs. 2 and 3). Representative histograms are displayed in Fig. 2, showing the typical quadrant analyses for each cathepsin. The isotype control staining for CD11c revealed that in the whole population the most highly expressed cathepsin was cathepsin D (89% of cells). Double staining for CD11c demonstrated that approximately 82% of the population were CD11c positive DC and that the majority of these stained positive also for cathepsin D. The frequency of CD11c positive and negative cells expressing the different cathepsins and the level of expression (MFI) of each cathepsin are displayed in Fig. 3 ($n=3-5$ independent experiments). All cathepsins were expressed in both CD11c positive and negative populations, but a higher frequency was recorded in the CD11c positive cells. Similarly, the level of

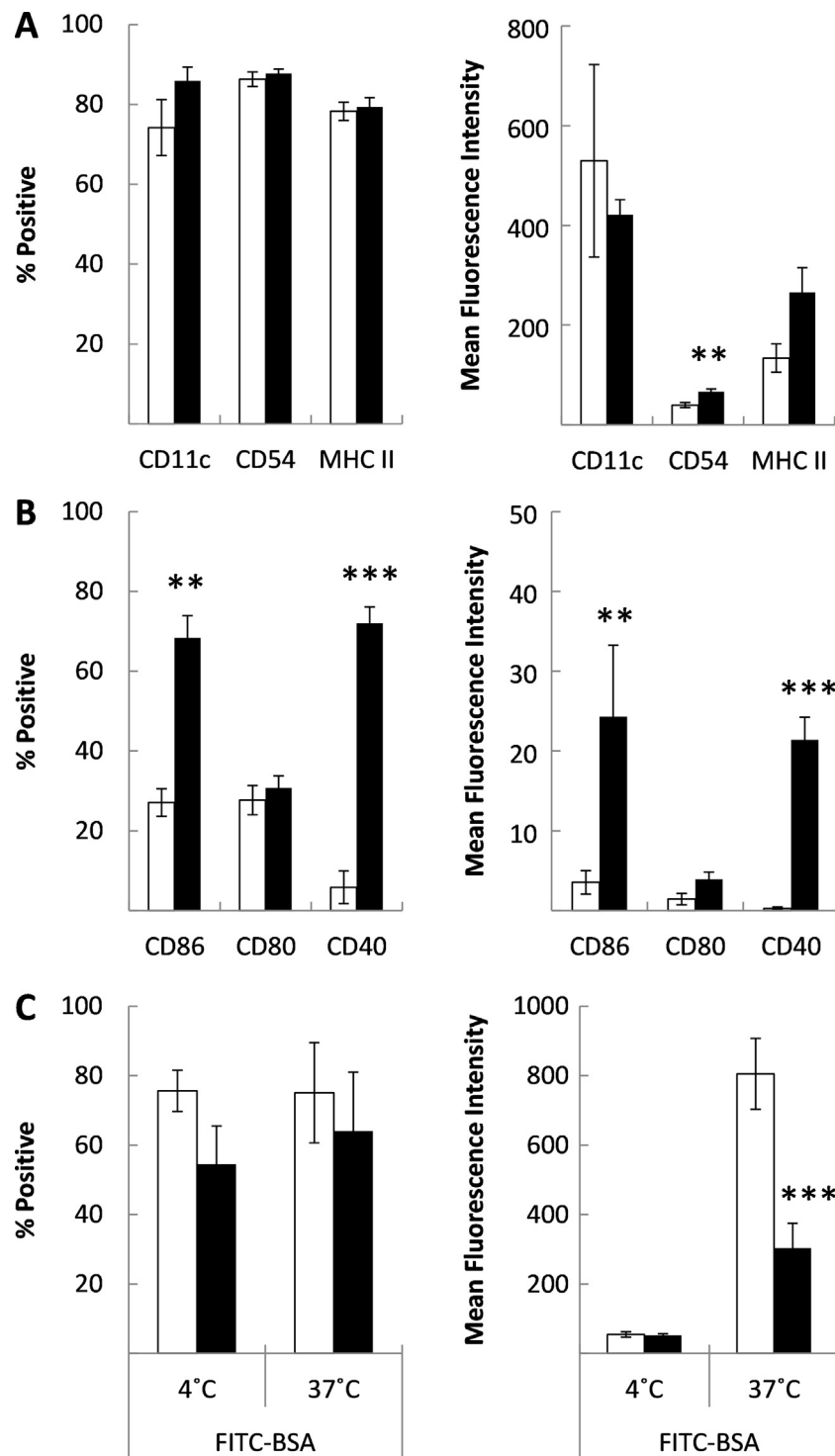


Fig. 1. Phenotype of resting and LPS activated day 12 BM-DC. DC were harvested on day 12 of culture and incubated for 24 h with LPS (100 ng/ml) (■), or medium alone (□). Membrane expression of the markers CD11c, CD54, MHC class II, CD86, CD80 and CD40 was assessed by flow cytometric analysis of 10^4 cells. Percentage of cells positive (A) and mean fluorescence intensity (MFI) (B) recorded for each membrane marker are shown. (C) Day 12 DC were pre-incubated for 1 h with 20 mM NaN_3 (■) or with medium alone (□) at 4 °C or 37 °C. Cells were incubated for a further 30 min at 4 °C or 37 °C in the presence of 1 mg/ml FITC-BSA and the extent of FITC-BSA uptake assessed by flow cytometry. The frequency of positive cells and the MFI are displayed. The mean $\pm$ standard error of 3–6 independent experiments is shown. The statistical significance of differences between medium alone and LPS-treated or NaN_3 -treated groups was assessed by two way ANOVA and Bonferroni post hoc tests (** $p < 0.01$; *** $p < 0.001$).

expression (MFI) was generally considerably higher in the DC population, with cathepsin D expression the most highly expressed molecule, followed by cathepsins B and L (data not shown). The percentage of cathepsin D positive cells was significantly higher ($p < 0.05$) than the percentage of cathepsin E and S positive cells in both the CD11c positive and negative populations. Furthermore,

this cathepsin was detected in >90% of the DC population. A similar pattern of expression was observed for the CD11c negative population, albeit at lower levels.

In subsequent experiments, the ability of recombinant cathepsin D, the most highly expressed DC cathepsin, to digest a range of different proteins (allergens and putative nonallergens; Table 1) was

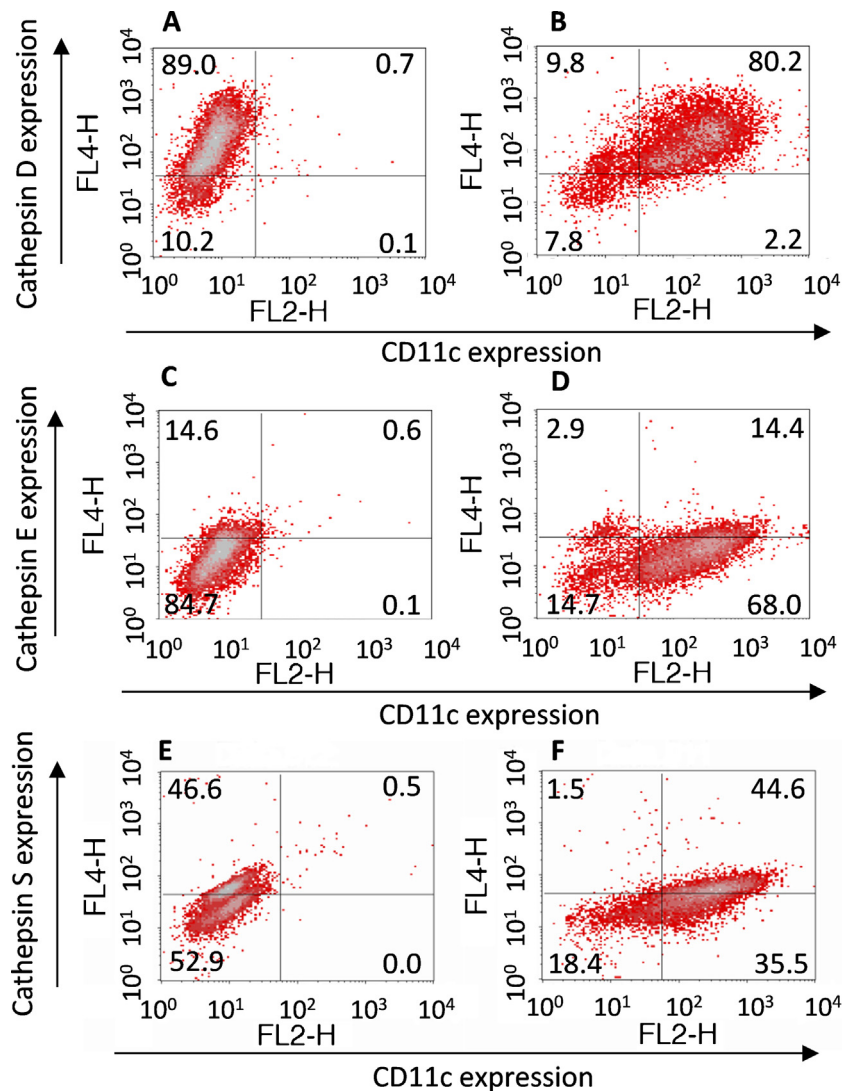


Fig. 2. Representative dot plots for the expression of intracellular cathepsins D, E and S by flow cytometry. DC were harvested on day 12 of culture, stained for surface CD11c expression, then permeabilised with saponin and stained for the intracellular expression of cathepsins D (A and B), E (C and D) or S (E and F). Cells (10^4) were analysed by flow cytometry and events are displayed on FL2 (FITC; CD11c) versus FL4 (AP; cathepsin) log dot plots. Representative dot plots are shown for FITC-isotype control/cathepsin stained populations (A, C and E) and for double stained CD11c-FITC/cathepsin populations (B, D and F).

compared with the ability of pepsin to digest those same proteins. Preliminary experiments were conducted using overnight incubation of cathepsin D with the reference proteins hemoglobin and ovalbumin; these identified the concentration of $30 \mu\text{g/ml}$ and the pH of 3.5 as optimal for protein digestion. Native or chemically reduced proteins were incubated at 37°C overnight alone or in the presence of $30 \mu\text{g/ml}$ recombinant mouse cathepsin D at pH 3.5. Control samples were incubated with cathepsin D and the inhibitor pepstatin A to demonstrate that the loss of protein bands was due to enzymatic digestion rather than nonspecific degradation due to low pH conditions, for example. The same proteins were incubated with pepsin under standard conditions (SGF; pH 1.2, and a 3:1 ratio of porcine pepsin to test protein solution) (Thomas et al., 2004) for up to 60 min. Following digestion proteins were run on SDS-PAGE gels and stained for protein and protein fragments using Coomassie blue. Representative gels for a cathepsin resistant protein (native β -lactoglobulin) and a cathepsin labile protein (native BSA) are illustrated in Fig. 4. It was not possible to visualise a band for cathepsin D as the levels of protein ($30 \mu\text{g/ml}$) were below the limit of detection for Coomassie blue staining. Bands for β -lactoglobulin and BSA were observed at 20 kDa and 70 kDa, respectively. There

was no loss of the β -lactoglobulin protein band following incubation with cathepsin D in the presence or absence of pepstatin A, whereas for BSA, the band for the whole protein disappeared completely and fragments of less than 10 kDa were detected in the presence of cathepsin D. Digestion of BSA by cathepsin D was completely inhibited by the presence of pepstatin A. For each protein, the extent of digestion of the main band by pepsin or cathepsin was quantified by densitometric scanning and is expressed as a function of the percentage protein remaining (Fig. 5, for $n=3$ independent experiments). The ability of both enzymes to digest bromelain was also investigated. However, under the conditions of both assays (buffer in the absence of enzyme), this allergen auto-digested to such an extent that it was not possible to determine additional effects of pepsin or cathepsin. The native allergens displayed a range of susceptibilities to digestion with pepsin; some were relatively stable with no significant loss of protein recorded over the 60 min time course (soy trypsin inhibitor, β -lactoglobulin, hens' egg lysozyme and actinidin). However, other allergens including the peanut allergens Ara h 1 and Ara h 2 and BSA, exhibited significant lability to pepsin within 0.5 min. With the exception of concanavalin A, the nonallergens were very rapidly digested by

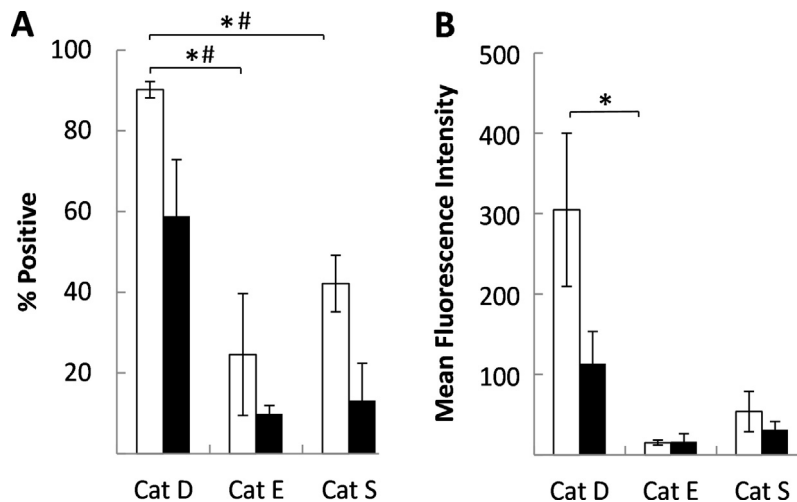


Fig. 3. Day 12 BM-DC expression of intracellular cathepsins. DC were harvested on day 12 of culture, stained for surface CD11c expression, then permeabilised with saponin and stained for the intracellular expression of cathepsins D, E and S. Cells (10^4) were analysed by flow cytometry. Cathepsin positive cells were characterised according to expression of CD11c. The frequency (A) and the mean fluorescence intensity (MFI) of expression (B) of cathepsin positive CD11c⁺ (□) and cathepsin positive CD11c⁻ (■) cells is displayed. Data from $n=3-5$ independent experiments are shown. The statistical significance of differences between levels of cathepsin D, E and S expression was assessed by one way ANOVA and Tukey's post hoc test for both CD11c⁺ (* $p<0.05$) and CD11c⁻ cells (# $p<0.05$).

pepsin, with significant loss of protein recorded within 0.5 min (Fig. 5). Chemical reduction of the proteins rendered both the allergens and the nonallergens considerably more labile to pepsin digestion, such that all proteins with the exceptions of actinidin and patatin and the nonallergen RUBISCO exhibited significant losses after 0.5 min and the majority were completely digested within 10 min (Table 2).

Most allergens were relatively resistant to digestion with cathepsin D, with no significant loss of protein recorded after overnight incubation with the exception of BSA and patatin which exhibited 80% and 60% loss of protein, respectively (Fig. 5). However, for some allergens the appearance of fragments was recorded (soy trypsin inhibitor and Ara h 1) (Table 2). Nonallergens were also relatively resistant to digestion with cathepsin D, with only hemoglobin displaying significant reduction in intact protein and the appearance of fragments following incubation of horse radish

peroxidase with the enzyme (Table 2). Chemical reduction of the proteins did not enhance the ability of cathepsin D digestibility to differentiate between nonallergens and allergens. Thus, significant digestion and/or the detection of fragments were recorded for all of the nonallergens with the exception of RUBISCO. In addition, following chemical reduction, the allergens soy trypsin inhibitor, β -lactoglobulin, ovalbumin, Ara h 1 and Ara h 2 were rendered susceptible to digestion with cathepsin, and for all of these allergens the presence of fragments was recorded (Table 2).

Finally, given the fact that endotoxin contamination can be a common feature of recombinant protein preparations (Zimmerman et al., 2006) that may be used for pepsin stability tests, the impact of exogenous endotoxin on the stability of selected proteins to digestion with pepsin and cathepsin D was examined. Despite the addition of 10^4 endotoxin units of LPS (equivalent to contamination of protein with 0.1% endotoxin) to the allergens ovalbumin

Table 2

A summary of the digestibility of the panel of allergens and putative nonallergens to pepsin and cathepsin D.

Test protein	Pepsin digestibility (min) (native protein)	Presence of pepsin resistant fragments (native protein)	Pepsin digestibility (min) (reduced protein)	Presence of pepsin resistant fragments (reduced protein)	Digestion by cathepsin (native protein)	Presence of cathepsin resistant fragments (native protein)	Digestion by cathepsin (reduced protein)	Presence of cathepsin resistant fragments (reduced protein)
Act d 1	>60 (>60)	N	20 (>60)	N	N	N	N	N
Ara h 1	0.5 (2)	Y	0.5 (2)	N	N	Y	Y	Y
Ara h 2	0.5 (5)	Y	0.5 (10)	N	N	N	Y	Y
BLG	30 (<60)	N	0.5 (2)	N	N	N	Y	Y
BSA	0.5 (0.5)	N	0.5 (5)	N	Y	Y	Y	Y
HEL	>60 (>60)	Y	0.5 (10)	N	N	N	N	N
OVA	5 (20)	N	0.5 (20)	N	N	N	Y	Y
Pat	0.5 (>60)	N	0.5 (>60)	N	Y	N	N	N
STI	>60 (<60)	Y	0.5 (5)	N	N	Y	Y	Y
Con A	30 (30)	N	0.5 (20)	N	N	N	N	Y
Hb	0.5 (2)	N	0.5 (0.5)	N	Y	Y	Y	Y
HRP	0.5 (2)	N	0.5 (2)	N	N	Y	Y	Y
RUBISCO	0.5 (10)	N	2 (>60)	N	N	N	N	N

The panel of native and reduced test proteins were incubated at 37 °C with SGF for 0–60 min or with cathepsin D for 16 h and samples analysed by SDS-PAGE as described in Fig. 5. Pepsin digestibility was defined as the first time period (in min) at which a statistically significant reduction in the protein could be detected on the SDS-PAGE gel and (in parentheses) the first time period at which <10% of the protein remained. The presence of pepsin resistant fragments after the final time point (60 min) is indicated by Y=yes or N=no. Cathepsin digestibility was defined as whether the enzyme induced a statistically significant reduction in the protein detected on the SDS-PAGE gel in comparison with the pepstatin treated control sample (Y=yes or N=no). The presence of cathepsin resistant fragments is indicated by Y=yes or N=no. Data for native and reduced proteins are shown.

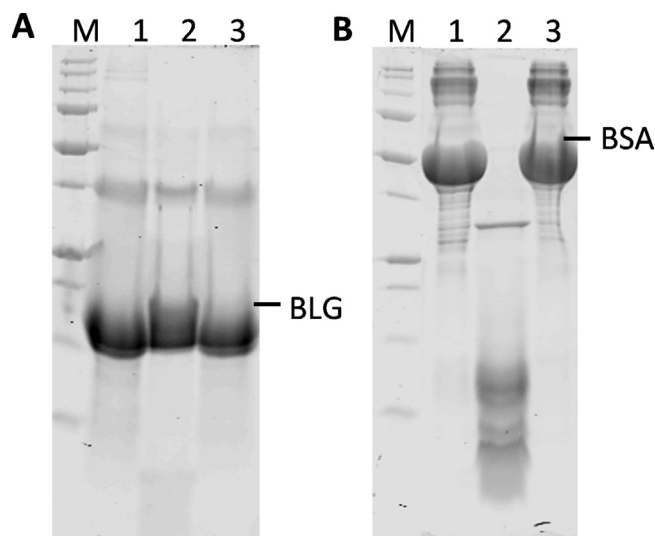


Fig. 4. Cathepsin D digestion of β -lactoglobulin (BLG) and bovine serum albumin (BSA): representative images. Proteins (BLG and BSA) were incubated at 37 °C with cathepsin D overnight (16 h). Control samples contained protein, cathepsin and the inhibitor pepstatin A. Samples were analysed using SDS-PAGE with a tricine buffer system for resolving small peptides, bands were stained using Coomassie brilliant blue. Molecular weight markers are indicated M on all gels, with molecular weights ranging from 10 to 250 kDa. Gels were loaded as follows: lane 1 test protein alone, lane 2 test protein with cathepsin D, lane 3 test protein with cathepsin D and the inhibitor pepstatin A. (A) BLG with cathepsin D. (B) BSA with cathepsin D.

and β -lactoglobulin and the nonallergens horse radish peroxidase and concanavalin A, there was no effect on the kinetics or extent of digestion by either enzyme (data not shown).

4. Discussion

It is over 15 years since the observation was made that there is an association between stability of proteins to digestion with pepsin and the potential for allergenicity (Astwood et al., 1996). Since then it has been found that this association is not an absolute phenomenon with stable nonallergens and unstable allergens identified (Fu et al., 2002; Lucas et al., 2008; Thomas et al., 2004). The main hypothesis investigated herein was that the association between stability and allergenic potential may reflect differences in intracellular processing and presentation of allergens/nonallergens by APC. One of the first aims was therefore to characterise the expression of proteolytic enzymes in a prototypic APC population, mouse BM-DC (Dearman et al., 2009). The BM-DC population utilised was shown to display a DC phenotype (CD11c, CD54 and MHC class II positive), but was relatively immature and could be induced to upregulate co-stimulatory molecules CD86 and CD40 upon activation with the DC stimulator LPS. Immature DC have been shown previously to be one of the few cell types to take up soluble antigen by macropinocytosis (internalisation of large [0.5–3 μ m] vesicles) and by receptor-mediated endocytosis, properties which equip them to capture and process antigen for subsequent presentation to T cells (Sallusto et al., 1995). Thus, endocytic activity of the BM-DC was explored using FITC-BSA, a molecule which is taken up by both mechanisms (Sallusto et al., 1995; Cherukuri et al., 1997). Importantly, the BM-DC population was shown to be very efficient at endocytosis; with high levels of uptake recorded after incubation at 37 °C for only 30 min. Consistent with an active metabolic process, uptake was inhibited markedly by culture at 4 °C or in the presence of NaN₃ (Oda and Maeda, 1986). Having established that the BM-DC in culture displayed an immature DC-like phenotype with active antigen uptake activity, the intracellular cathepsin expression profile was investigated by flow cytometry.

A range of cathepsins have been suggested to be important for antigen processing by DC including cathepsins D, E, S, B, L, H, F, Z, V, O, C and possibly K (Watts, 2001; Fineschi and Miller, 1997; Honey and Rudensky, 2003; Villadangos et al., 1999; Colbert et al., 2009). Cathepsins D, E, S, B and L were selected for study on the basis of being most strongly implicated as having specific roles in antigen processing, (Van Noort and Van Der Drift, 1989; Rodriguez and Diment, 1992; Hewitt et al., 1997). Consistent with the findings of Egger et al. (2011), who used mass spectrometry to demonstrate that these cathepsins were present in the lysosomal fraction of BM-DC, all 5 proteins were detected, with cathepsin D being the most highly expressed.

Having identified cathepsin D as the most highly expressed family member in BM-DC, the lability of a range of allergens and nonallergens to digestion by this enzyme was compared with pepsin digestibility. The allergens selected included plant and animal proteins and encompassed a range of potencies: several peanut allergens (Ara h 1 and 2), proteins that are associated with long lasting and potentially severe reactions, and patatin and BSA, relatively rare allergens (Ballmer-Weber and Hoffman-Sommergruber, 2011; Traidl-Hoffmann et al., 2009; Radauer and Breiteneder, 2007). The choice of nonallergenic proteins was more restricted. Although there is a general consensus that of the thousands of foreign proteins to which we are exposed only a small fraction are important allergens (Traidl-Hoffmann et al., 2009; Radauer and Breiteneder, 2007), there is much less agreement regarding those proteins that may be truly lacking in allergenicity. The main concern is that for most proteins that have not been identified as being allergens, rather than there being firm evidence of lack of allergenic potential, there is merely absence of evidence that such proteins are allergenic (possibly due to insufficient exposure in the human population). The 4 proteins that have been selected as nonallergens (concanavalin A, horse radish peroxidase, RUBISCO and hemoglobin) have been used previously as putative negative control proteins in tests for allergenicity (Astwood et al., 1996; Fu et al., 2002; Thomas et al., 2004). In order to facilitate comparisons with published data, the standardised conditions for the pepsin digestibility outlined in the multilaboratory comparison were utilised (Thomas et al., 2004).

As summarised in Table 2, the majority of the known allergens were largely pepsin-stable with more than 10% of the whole protein remaining after 60 min with pepsin (5 out of 9). However, two of the most potent allergens (Ara h 1 and Ara h 2) were relatively easily digested by pepsin, as were BSA and ovalbumin; this is consistent with previous publications in which all 4 allergens were completely digested within 5 min (Fu et al., 2002; Thomas et al., 2004). Three out of 4 of the nonallergens were rapidly digested by pepsin (less than 10% remaining after 10 min) with concanavalin A being the most resistant with 10% of the whole protein remaining 30 min. Thomas et al. (2004) also found concanavalin A to be relatively resistant to digestion with pepsin. In contrast, the same panel of proteins in their native form were all relatively stable to digestion with cathepsin D, with the exception of BSA and patatin (which are regarded as less potent allergens) and the non-allergen hemoglobin which is a known substrate of cathepsin D and as such confirms the activity of the enzyme used in this series of experiments (Brindley et al., 2001). Per force the conditions of the pepsin and cathepsin digestion assays are different with respect to pH (pH 1.2 and pH 3.5 being optimal, respectively). It is also of note that the incubation times are 1 h and 16 h, respectively, and that there is a 80-fold difference in the ratio of enzyme to test protein (3:1 and 1:27, respectively).

Given the lack of activity of the cathepsin on the majority of native protein allergens in the panel, the influence of chemical reduction on the digestibility of proteins was investigated also. Chemical reduction of the proteins helps to unfold their 3D structure and expose their catalytic sites (Van Noort and Jacobs, 1994;

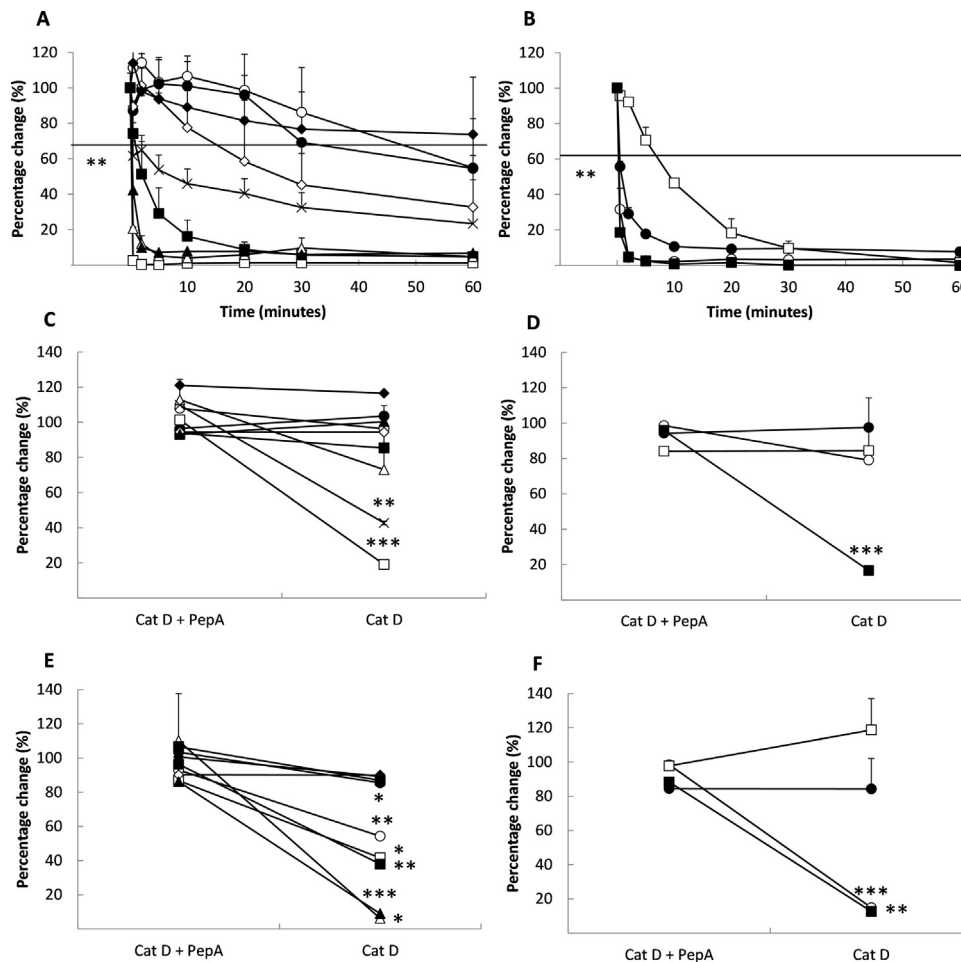


Fig. 5. Pepsin digestibility of native proteins and cathepsin D digestibility of native or chemically reduced proteins. Allergens (A) or nonallergens (B) were incubated at 37 °C with pepsin (SGF) for 0–60 min. The same panel of proteins were untreated (C, D; native) or were pretreated with 2BME and urea for 2 h at 37 °C (E, F; reduced) and incubated at 37 °C for 16 h with cathepsin D in the presence or absence of pepstatin A. Samples were analysed by SDS-PAGE gel electrophoresis as described in Fig. 4. Bands were quantified using a li-cor gel scanner and Odyssey software. The integrated intensity values for each band were used to calculate the percentage change in intensity for each of the samples in comparison with the 0 min time point for the pepsin digestion assay and in comparison with the protein alone sample for the cathepsin D assay. The mean and SE percentage intensity change for 3 independent experiments are shown for allergens (A, C, E; soybean trypsin inhibitor, ○; β-lactoglobulin, ●; BSA, □; ovalbumin, ■; hens' egg lysozyme, ◇; actinidin, ◆; Ara h 1, △; Ara h 2, ▲; and patatin, ×) and nonallergens (B, D, F; horse radish peroxidase, ○; RUBISCO, ●; hemoglobin, ■ and concanavalin, □). For the pepsin digestion assay one-way ANOVA and post hoc Dunnett's test was conducted in comparison with the zero time point for each test protein (** $p < 0.01$). All points below the horizontal line were significantly decreased with the exceptions of soybean trypsin inhibitor, hens' egg lysozyme and actinidin (A). For the cathepsin D assay one-way ANOVA and post hoc Dunnett's test was conducted in comparison with the test protein alone sample for each test protein (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Toda et al., 2011). Importantly, reducing conditions are found in the endosomal and lysosomal compartments of DC (Arunachalam et al., 2000) and therefore reduced components may be in a conformation more representative of the proteins which would encounter cathepsin D physiologically. Reduced proteins were clearly much more sensitive to digestion by cathepsin, with 6 of the allergens and 2 of the nonallergens showing significant loss of the whole protein. However, chemical reduction did not improve the ability of cathepsin D digestibility to discriminate between allergens and nonallergens. A similar impact of reduction was noted for pepsin digestibility, with most reduced proteins being more labile than their native counterparts, although such has less physiological relevance, given that there is no evidence for reduction in the natural environment of pepsin.

It has been suggested previously that the production of fragments may be a useful metric in the interpretation of the digestibility of proteins (Thomas et al., 2004). It is assumed that a certain minimum size of protein is required for interaction with the immune system and the initiation and development of a specific immune response. For the elicitation phase of IgE antibody responses (mast cell degranulation) the minimum size of protein

has been estimated to be 3–5 kDa; the size needed to span the gap between IgE molecules on the surface of mast cells (Van Beresteijn et al., 1995). If the production of proteolysis resistant fragments is taken into account, then for pepsin this consideration allows further discrimination between the selected allergens and nonallergens in their native form. Thus not only are 3 out of the 4 nonallergens relatively pepsin-labile, but also there are no pepsin-resistant fragments observed, whereas for the allergens, 5 out of 9 were relatively stable and for 2 of the labile allergens (Ara h 1 and 2), persistent fragments were recorded. For cathepsin D there was no further discrimination between proteins achieved by consideration of resistant fragments: for native and reduced proteins, fragments were detected in both the allergens and nonallergens.

Taken together these data confirm that there is a general relationship between resistance to digestion with pepsin and allergenicity, particularly when the production of pepsin resistant fragments is taken into account. The relationship is not absolute, but the information gained from the assay does provide useful information in a weight of evidence approach for the assessment of allergenicity. Although cathepsin D is the most abundant cathepsin

detected in antigen processing BM-DC, this enzyme is not an appropriate substitute for pepsin in the protein stability assay as it shows little discrimination between allergens and nonallergens. The hypothesis that pepsin stability may be a surrogate for stability to digestion within antigen processing cells may still hold true, but the use of a single enzyme is an oversimplification. There is evidence to suggest for example that different proteins are targeted by different proteolytic enzymes within DC: tetanus toxoid by the cysteine protease asparagine endopeptidase, Bet v 1 (the pollen allergen) by cathepsin S whereas ovalbumin is a substrate for both cathepsin D and E (Egger et al., 2011; Free et al., 2006). An alternative approach, although considerably more technically demanding than simply utilising recombinant enzymes, is to isolate DC endosomal lysates that contain the full complement of proteolytic enzymes in the appropriate ratios and to use these to examine the stability of test proteins to digestion. This type of approach has shown some success in identifying those proteins that are more immunogenic than others (those that were more stable to digestion) (Egger et al., 2011) and may be applicable to allergenicity testing.

Conflict of interest

There is no conflict of interest to declare.

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