



# *In vitro* pepsin resistance of proteins: Effect of non-reduced SDS-PAGE analysis on fragment observation



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## ABSTRACT

The introduction of novel proteins to food products carries with it the need to assess the potential allergenicity of such materials. Resistance to *in vitro* pepsin digestion is one parameter considered in such a risk assessment using a weight of evidence approach; however, the methodology used to investigate this has not been fully standardised. *In vitro* pepsin resistance assays typically involve SDS-PAGE performed under reducing conditions, with limited published data available on the assay using non-reducing conditions despite the need to consider non-reducing analysis techniques having been highlighted by regulatory bodies such as the European Food Safety Authority (EFSA). The purpose of the work reported here was to investigate the applicability of (and additional insight provided by) non-reducing analyses, by digesting a set of proteins using a ring-trial validated method, with analysis by SDS-PAGE under both reducing and non-reducing conditions. *In silico* prediction of digest fragments was also investigated. Significant differences were observed between results under reduced and non-reduced conditions for proteins in which disulphide bonds have a major role in protein structure, such as ribulose 1,5-diphosphate carboxylase (RUBISCO) and bovine serum albumin. For proteins with no or few disulphide bonds, no significant differences were seen in the results. Structural information such as disulphide bond numbers and positions should be considered during experimental design, as a non-reduced approach may be appropriate for some proteins. The *in silico* approach was a useful tool to suggest potential digest fragments, however the predictions were not always confirmed *in vitro* and should be considered a guide only.

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## 1. Introduction

The introduction of novel proteins to food products carries with it the need to assess the potential allergenicity of such materials and this is currently undertaken using a weight of evidence approach, consistent with consultation reports and guidelines published by expert scientific bodies including the Food and Agriculture Organisation of the United Nations/World Health Organisation (FAO/WHO [1]), Codex Alimentarius Commission (Codex [2]) and the

European Food Safety Authority (EFSA [3]). *In vitro* resistance to pepsin digestion is one of the pieces of evidence for such an assessment with consideration given to both the speed of digestion and the size of fragments formed. Currently little is known about the relationship between pepsin resistance and ability to sensitise, but there is a clear rationale for a correlation with the potential to elicit allergic reactions *via* ingestion, including *in vivo* evidence (animal and human) that there is a minimum peptide size able to elicit a Type 1 allergic response in the order of 2–3 kDa (Poulsen and Hau [4], Van Hoeyveld et al. [5], and Terheggen-Lagro et al. [6]).

It is generally recognised that variations in assay conditions employed can have a large impact on the digestibility

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**Table 1**  
Protein information.

Protein	UnitProtKB accession number	Approximate protein mass (kDa)	Disulphide bonds
β-Lactoglobulin	P02754	19.9	2
Bovine serum albumin	P02769	69.3	17
Concanavalin A	P02866	31.5	None
RUBISCO	P00875 (large chain) Q43832 (small chain)	52.7 (Large)	4
		20.3 (Small)	
		584 (Whole protein)	Large subunits present in dimers formed by S—S bonds
		8 Large and 8 small subunits present in whole protein.	
Horseradish peroxidase	P00433	38.8–44 (Glycosylated)	4
Ovalbumin	P01012	42.9	1
β-Casein	P02666	25.1	None

of proteins *in vitro* and a ring-trial validated protocol for pepsin resistance assays has subsequently become a *de facto* standard method [7].

In recent years, attempts have been made to develop approaches and models that aim to closely replicate human *in vivo* digestion, however these have not been systematically assessed to determine if they are more predictive of allergenic potential. Thus the method proposed by Thomas et al. is used in-house by this group to provide information on the pepsin resistance of proteins for risk assessment purposes. The method proposed by Thomas et al. [7] does not (nor does it seek to) replicate human *in vivo* digestion but serves as a standardised (at least partially) method for comparing the pepsin resistance of proteins in a well understood context.

Analysis for samples from this assay is generally carried out using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). However the standard protocol for SDS-PAGE is performed under reducing conditions in which all disulphide bonds are reduced prior to analysis. This may lead to the observed digestion products appearing smaller than they are under non-reducing conditions due to the breaking of disulphide bonds holding multiple fragments together. This may not accurately reflect the situation *in vivo*, as disulphide bonds may not be broken in the stomach. In addition, larger fragments originally held together by disulphide bonds may be too small to resolve under SDS-PAGE analysis following reduction and may not be observed at all.

Disulphide bonds are an important structural feature in several well known plant allergen families, as discussed by Hauser et al. [8], and allergens from several families (2S albumins, chitinases) have been shown to retain allergenicity following pepsin digestion, even when appearing extensively digested under reducing SDS-PAGE analysis.

This suggests that for proteins with numerous disulphide bonds, the accepted analysis method may not be appropriate, as large disulphide-linked fragments would not be detected. Indeed this was recognised in a recent EFSA Opinion [3] that recommended 'If the test protein contains disulphide bridges, the presence of potential larger fragments containing re-associated disulphide-bonded fragments should be verified by isolation and determination under non-reducing conditions.' The potential use of both reducing and non-reducing conditions for SDS-PAGE for *in vitro* pepsin resistance assays, and need for

careful consideration of method details and data interpretation when using SDS-PAGE was also discussed by Mills et al. [9]. Despite this there are few examples in the published literature of non-reduced SDS-PAGE analysis being performed when studies of resistance to pepsin digestion are carried out. Moreno et al. [10] studied the stability of Brazil nut 2S albumin (Ber e 1) under both reducing and non-reducing conditions, and concluded that the conserved skeleton of cysteine residues played a critical role in maintaining the protein's core structure during proteolysis. Koppelman et al. [11] also studied Ber e 1 and demonstrated that reduction and alkylation of cysteine residues prior to treatment with pepsin rendered the protein highly susceptible to hydrolysis, highlighting the key role of the disulphide bridge in protein stability. Koppelman et al. [12] also studied a range of peanut allergens and demonstrated that proteolysis for key allergens Ara h 2 and Ara h 6 could only be demonstrated under reducing conditions.

Taking all of this into account, the aim of this study was to highlight some methodological considerations around the pepsin resistance assay and its interpretation in the light of allergenicity prediction, and suggest some refinements to improve the value of the information it generates. More specifically the study aimed to investigate the applicability of (and additional insight provided by) non-reducing analyses, by digesting a selection of proteins using a ring-trial validated method, with analysis by SDS-PAGE under both reducing and non-reducing conditions.

## 2. Materials & methods

### 2.1. Pepsin activity

A ratio of 10U of pepsin activity/μg test protein was used throughout the study, as described by Thomas et al. [7]. Pepsin from porcine gastric mucosa (product number P7012) was purchased from Sigma-Aldrich Company Ltd (Dorset, UK) for use in the study, with activity of 2913 U/mg protein as analysed by the supplier. The ratio of pepsin/protein in the digestion incubate was approximately 3:1 (w:w).

### 2.2. Test proteins

The majority of test proteins chosen for this study were from the list of proteins studied by Thomas et al. [7],

and all were sourced from Sigma-Aldrich. The proteins studied were bovine  $\beta$ -lactoglobulin (BLG, product number L0130), bovine serum albumin (BSA, product number A0281), concanavalin A from Jack beans (ConA, product number C2010), ribulose 1,5-diphosphate carboxylase from spinach leaves (RUBISCO, product number R8000), horseradish peroxidase (HRP, product number P6782) and chicken ovalbumin (Ova, product number A5503)

An additional protein, the common milk allergen  $\beta$ -casein (BCas, product number C6905), not used in the Thomas study was included to widen the set of proteins studied.

A summary of information regarding the test proteins, including protein mass and disulphide bond numbers (sourced from UniProt Knowledge Base, [www.uniprot.org](http://www.uniprot.org)) and allergen status according to Thomas et al. [7] is given in Table 1.

### 2.3. Pepsin resistance assay conditions

A single pH (pH2) was used for the digestion of all the proteins in this study, to maximise the formation of fragments.

For each test protein, vials were prepared for each time-point containing pepsin solution ( $\sim 5.5$  mg/ml in 0.06 M sodium chloride, pH2) and incubated at 37 °C. Test protein solution ( $\sim 8$  mg/ml in ultrapure water (MilliQ system, Millipore, Watford, UK), except RUBISCO which was prepared in 50 mM Tris-HCl pH9.5 due to solubility constraints) was added, such that the final concentrations in the incubates were approximately 4 mg/ml pepsin and 1.3 mg/ml test protein (3:1 (w:w)). At each timepoint (30 s, 2 min, 5 min, 10 min, 20 min, 30 min and 60 min) samples ( $n=2$ ) were stopped with sodium carbonate (0.8 M, 0.57 ml/ml incubate) and neutralised with concentrated hydrochloric acid (50% solution, 0.075 ml/ml incubate), to give a final concentration of approximately 0.8 mg/ml test protein. T0 samples were prepared by the addition of sodium carbonate to pepsin solution prior to the addition of the test protein solution. A reagent blank was also prepared, using ultrapure water in place of the test protein solution. A test protein standard was prepared for analysis alongside the samples, by diluting the test protein solution to approximately 0.8 mg/ml.

### 2.4. SDS-PAGE electrophoresis

Digest samples and the reagent blank and standard samples were further diluted 1 in 4 in reducing or non-reducing sample buffer (XT sample buffer (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK)  $\pm 100$  mM dithiothreitol) prior to SDS-PAGE electrophoresis according to the method of Schagger and von Jagow [13]. Reduced and non-reduced samples were run on separate gels, however for each gel a standard of the opposite reduction status was run alongside the samples, to allow an on-gel comparison of the migration of the reduced/non-reduced parent protein.

Prior to application to the gel, all samples were heated in a boiling water-bath for 5 min. Then 10  $\mu$ l of prepared solutions were applied to the appropriate lanes of a Criterion Peptide 12+2 well 10–20% linear

gradient gel (Bio-Rad) and electrophoresis performed using Tris-Tricine-SDS running buffer. Molecular weight markers (Invitrogen Novex sharp markers (3.5–260 kDa, (Life Technologies Ltd., Paisley, UK)) & Bio-Rad polypeptide SDS-PAGE standard (1.4–26.6 kDa, Bio-Rad)) were run concurrently on all gels to enable molecular weight determination. The Bio-Rad polypeptide standard was diluted in reducing sample buffer prior to application to gels for both analysis conditions. Novex sharp markers were applied to the gel undiluted. Molecular weights for non-reduced samples calculated against reduced standards should be considered estimations only, as intact disulphide bonds can alter the shape of the molecule and therefore the apparent molecular weight under SDS-PAGE conditions.

### 2.5. Gel staining

#### 2.5.1. 1X Krypton total protein staining

For all samples the gels were fixed in aqueous 40% (v:v) ethanol/10% (v:v) acetic acid for two 30 min periods, before washing in ultrapure water for 5 min. The gels were stained overnight in aqueous 1X Krypton (Thermo Scientific (Pierce), Hemel Hempstead, UK) stain before destaining with aqueous 5% (v:v) acetic acid for a minimum of 5 min. Finally the gels were washed in ultrapure water for two 15 min periods. All staining steps were performed under gentle agitation using an orbital mixer.

#### 2.5.2. Coomassie Blue R-250 gel staining

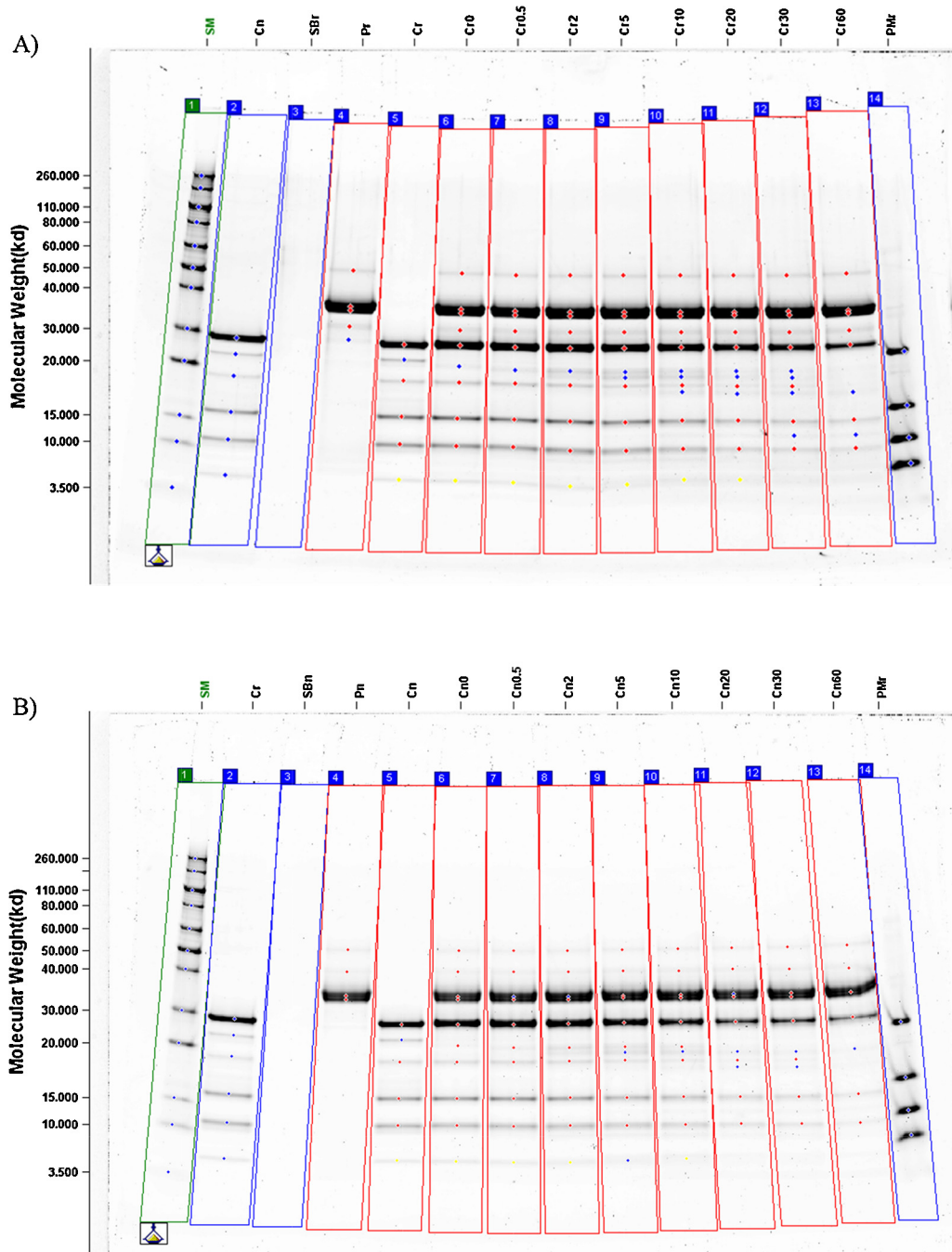
For BSA a repeat analysis was performed using Coomassie Blue staining. The gels were fixed in aqueous 40% (v:v) methanol/10% (v:v) acetic acid for 1 h before staining overnight in neat Coomassie Brilliant Blue R-250 stain (Bio-Rad). The stained gels were then destained in two changes of aqueous 40% (v:v) methanol/10% (v:v) acetic acid, for 15 min, until a satisfactory background was achieved, before washing in ultrapure water for 1 h. All staining steps were performed under gentle agitation using an orbital mixer.

#### 2.5.3. Gel imaging and analysis

Fluorescently stained (Krypton) gel images were captured using the Typhoon Trio+ variable mode imager and Scanner control software. Coomassie Blue stained gels were captured using the Imagescanner II with Labscan software (version 5). The gel images were analysed using TotalLab TL120 software.

### 2.6. In silico pepsin proteolysis

In silico analysis of the potential digestion fragments for RUBISCO, HRP and BSA was performed using Peptide Cutter ([http://web.expasy.org/peptide\\_cutter/](http://web.expasy.org/peptide_cutter/)). The potential fragments of pepsin digestion were examined in the context of the sequence to determine whether the resulting peptides could be linked by disulphide bonds to form larger fragments, using disulphide bond position information taken from the UniProt Knowledge Base entry for each protein.

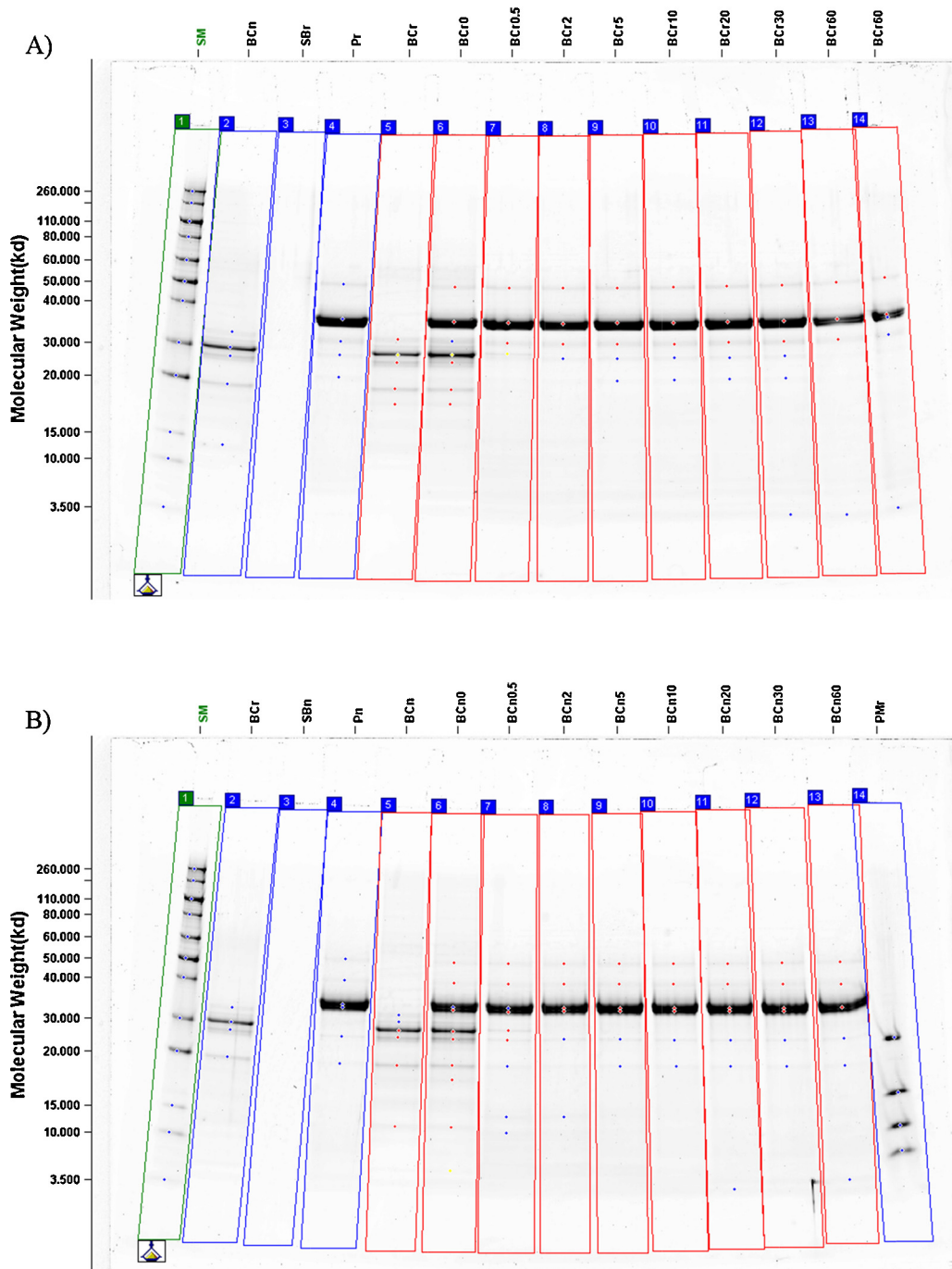


**Fig. 1.** Reduced (A) and non-reduced (B) analyses of ConA. From left to right for each gel the lanes contain: Novex sharp markers (Lane 1), test protein blank (opposite reduction status to digest samples) (Lane 2), sample buffer (Lane 3), pepsin blank (Lane 4), test protein blank (same reduction status as digest samples) (Lane 5), digest samples (Timepoints: T0, 0.5, 2, 5, 10, 20, 30, 60 min) (Lanes 6–13) and polypeptide SDS-PAGE standards (Lane 14).

### 3. Results and discussion

The images obtained for each test protein under reduced and non-reduced analysis conditions were compared, and differences in the distribution and lifespan of any fragments formed was noted and interpreted with reference to information available about protein structure, and disulphide bond arrangements (Table 1). The selected proteins

had a variety of disulphide bond arrangements; none (ConA, BCAs), few (1 or 2, Ova, BLG) and many (4+, BSA, RUBISCO, HRP). The following proteins originally used by Thomas et al. [7] were not included in this work: Ara h 2 could not be commercially sourced, and was omitted as the non-reduced analysis of its digestion fragments had already been published by Koppelman et al. [12]. Phosphinothricin acetyltransferase (PAT) could also not be



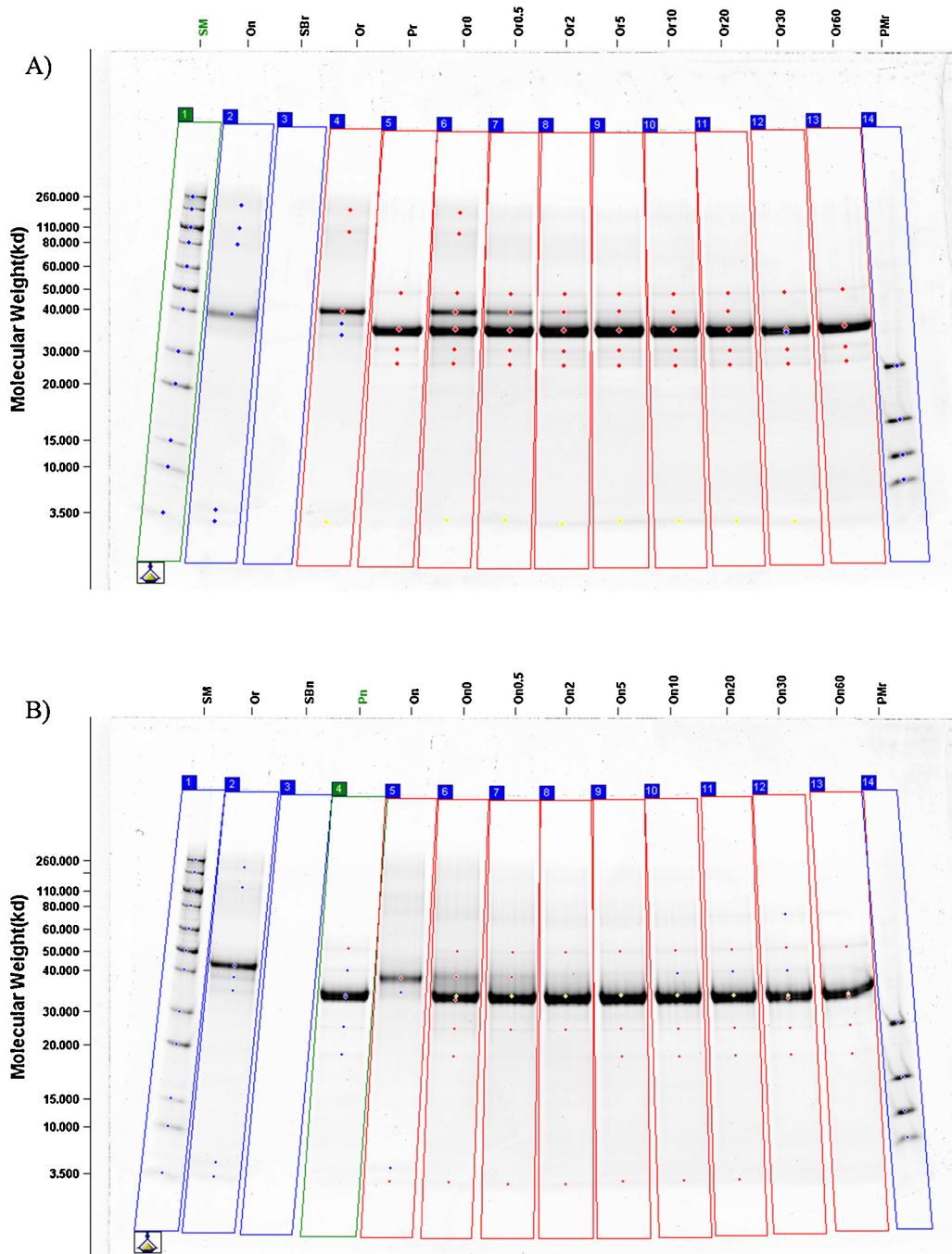
**Fig. 2.** Reduced (A) and non-reduced (B) analyses of BcAs. From left to right for each gel the lanes contain: Novex sharp markers (Lane 1), test protein blank (opposite reduction status to digest samples) (Lane 2), sample buffer (Lane 3), pepsin blank (Lane 4), test protein blank (same reduction status as digest samples) (Lane 5), digest samples (Timepoints: T0, 0.5, 2, 5, 10, 20, 30, 60 min) (Lanes 6–13) and polypeptide SDS-PAGE standards (Lane 14).

commercially sourced. Kunitz soybean trypsin inhibitor (STI) was not studied as it was extremely resistant to digestion, with limited fragment formation observed in the Thomas study. In addition, ovomucoid was also omitted, due to analysis problems reported in the Thomas study.

### 3.1. Comparison of reduced and non-reduced analyses for test proteins

#### 3.1.1. No or few disulphide bonds

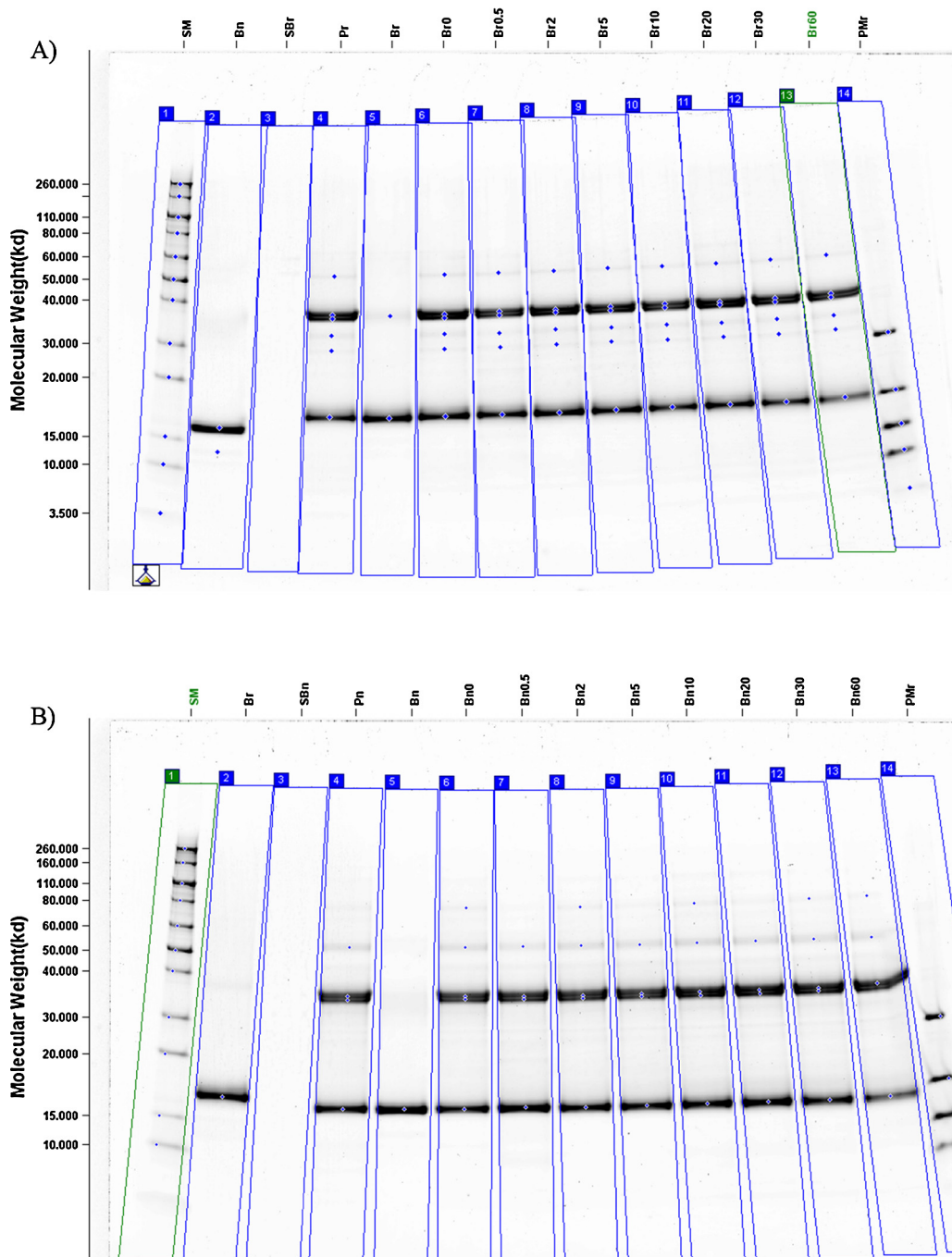
BcAs and ConA have no disulphide bonds, therefore no differences were expected between the analyses. These



**Fig. 3.** Reduced (A) and non-reduced (B) analyses of Ova. From left to right for each gel the lanes contain: Novex sharp markers (Lane 1), test protein blank (opposite reduction status to digest samples) (Lane 2), sample buffer (Lane 3), pepsin blank (Lane 4), test protein blank (same reduction status as digest samples) (Lane 5), digest samples (Timepoints: T0, 0.5, 2, 5, 10, 20, 30, 60 min) (Lanes 6–13) and polypeptide SDS-PAGE standards (Lane 14), except for gel (A) in which the pepsin blank and reduced Ova standard were reversed.

proteins were used as negative controls to verify whether the use of a non-reducing sample buffer would affect the gel images obtained. Ova and BLG have 1 and 2 disulphide bonds respectively, and only limited differences were expected. For all four proteins no significant differences were observed between the gel images for reduced and non-reduced analyses (Figs. 1–4). Where digestion

fragments were observed (ConA, Ova, BCAs) no significant differences in the appearance and lifespan of the fragments were noted. Despite being a known allergen, BCAs was rapidly digested with no significant fragments remaining at the end of the digestion time course suggesting that its immunological activity may be based on aggregates of smaller fragments, as discussed by Benedé et al. [14].

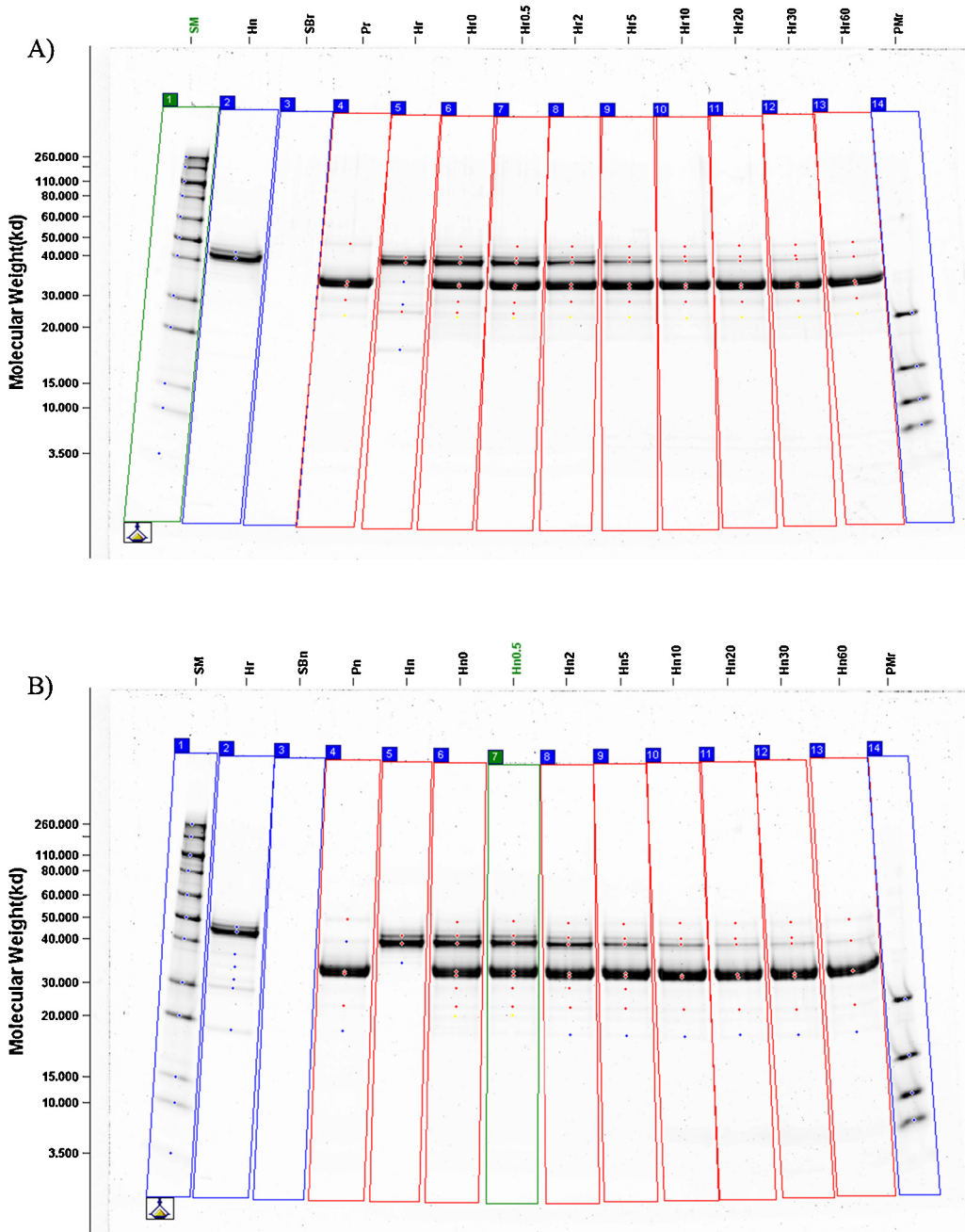


**Fig. 4.** Reduced (A) and non-reduced (B) analyses of BLG. From left to right for each gel the lanes contain: Novex sharp markers (Lane 1), test protein blank (opposite reduction status to digest samples) (Lane 2), sample buffer (Lane 3), pepsin blank \* (Lane 4), test protein blank (same reduction status as digest samples) (Lane 5), digest samples (Timepoints: T0, 0.5, 2, 5, 10, 20, 30, 60 min) (Lanes 6–13) and polypeptide SDS-PAGE standards (Lane 14). \* BLG was added to the pepsin blank samples in error.

It was noted that Ova was digested more rapidly than had been reported by Thomas et al. [7], and there was no evidence of the fragment formation observed in that earlier work. This could be due to the use of a different staining method in the current study, however a similar digestion profile for Ova was reported by Dearman et al. [15].

### 3.1.2. Many disulphide bonds

No major differences were observed between reduced and non-reduced analysis of HRP (Fig. 5), despite the parent protein containing four disulphide bridges. No significant digestion fragments were observed. It appears that the protein is primarily cleaved by pepsin in positions such that the fragments formed are not held together



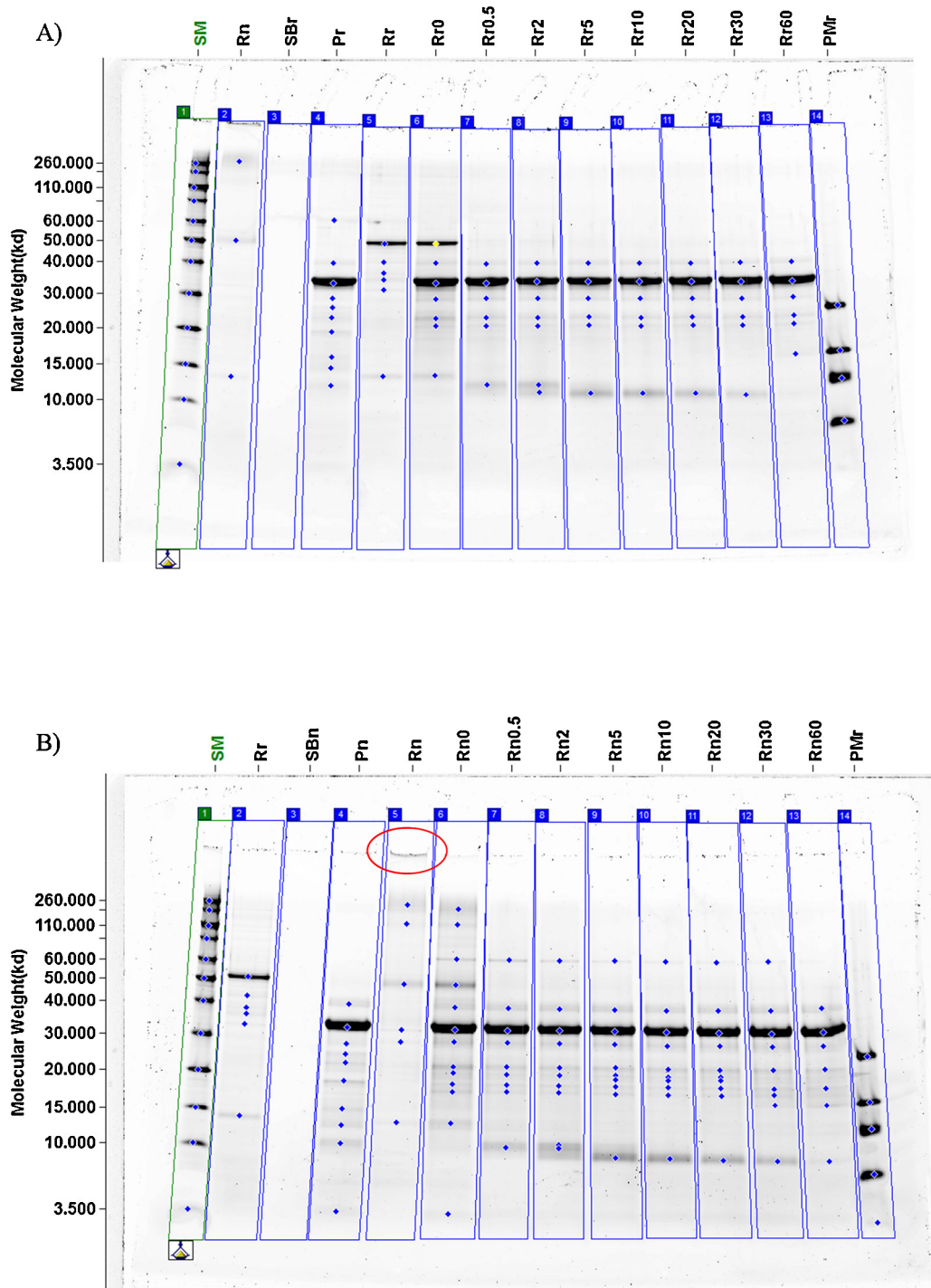
**Fig. 5.** Reduced (A) and non-reduced (B) analyses of HRP. From left to right for each gel the lanes contain: Novex sharp markers (Lane 1), test protein blank (opposite reduction status to digest samples) (Lane 2), sample buffer (Lane 3), pepsin blank (Lane 4), test protein blank (same reduction status as digest samples) (Lane 5), digest samples (Timepoints: T0, 0.5, 2, 5, 10, 20, 30, 60 min) (Lanes 6–13) and polypeptide SDS-PAGE standards (Lane 14).

by S–S bonds and give rise to only a very minor difference between the reduced and non-reduced analyses. Analysis of the predicted digestion from Peptide Cutter suggested that three potential fragments consisting of smaller peptide sequences held together by disulphide bonds could be formed following pepsin hydrolysis, with the largest containing 55 amino acids (~6 kDa). No evidence of these potential fragments was seen in the experimental data. It was noted that the parent protein

appeared more persistent than in the digestion assay performed by Thomas et al. [7], with a faint band still visible following 60 min of digestion, under both reducing and non-reducing conditions. No other evidence regarding *in vitro* HRP pepsin resistance could be located in the literature.

In contrast, RUBISCO and BSA both demonstrated significant differences between reduced and non-reduced analyses (Figs. 6–8).

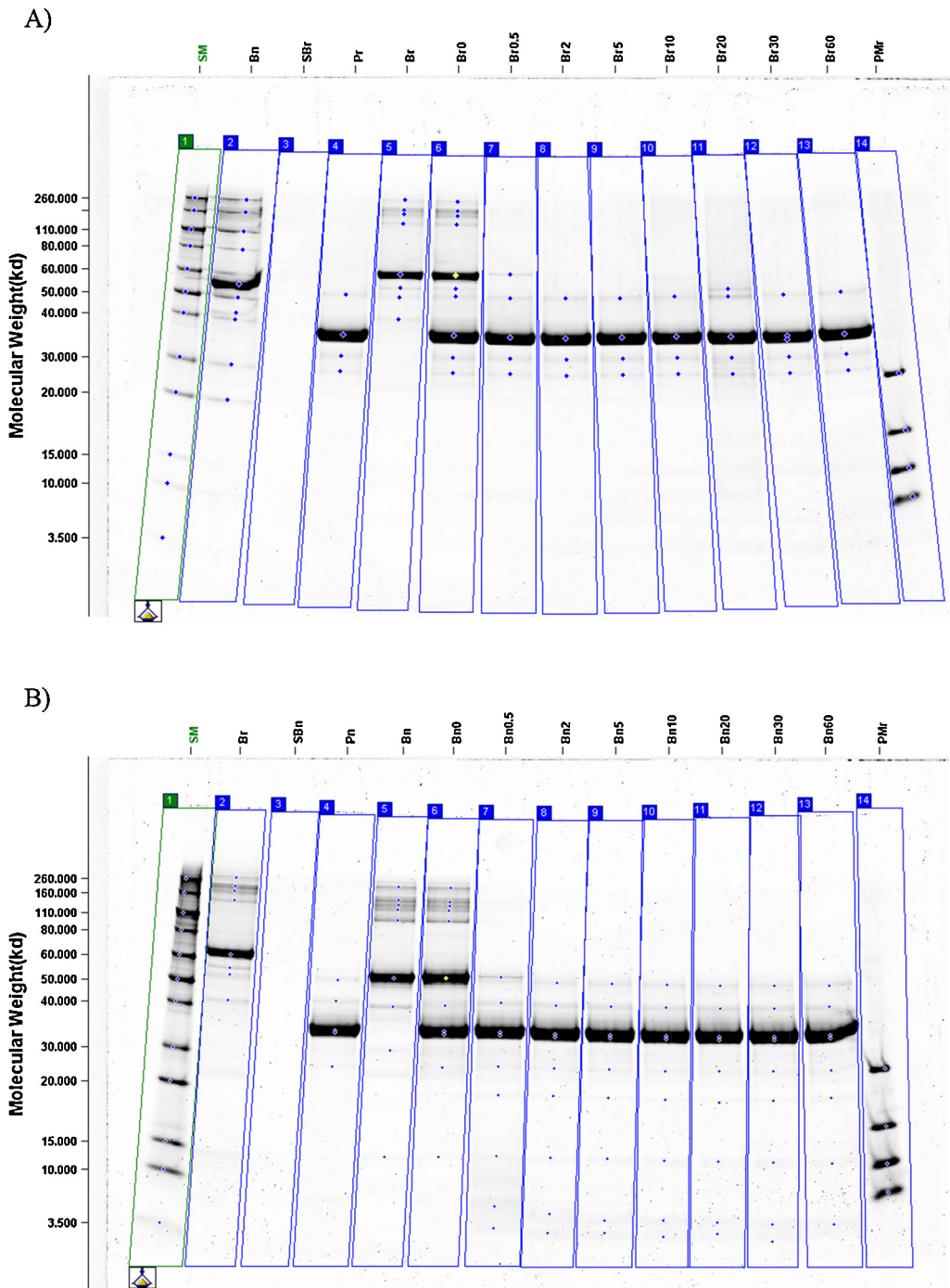




**Fig. 6.** Reduced (A) and non-reduced (B) analyses of RUBISCO. From left to right for each gel the lanes contain: Novex sharp markers (Lane 1), test protein blank (opposite reduction status to digest samples) (Lane 2), sample buffer (Lane 3), pepsin blank (Lane 4), test protein blank (same reduction status as digest samples) (Lane 5), digest samples (Timepoints: T0, 0.5, 2, 5, 10, 20, 30, 60 min) (Lanes 6–13) and polypeptide SDS-PAGE standards (Lane 14). Intact parent band highlighted in non-reduced image.

For RUBISCO bands were seen for both the large and small subunits (~53 and 20 kDa) in the standard and T0 samples under reducing conditions. Although the protein itself was rapidly digested, with the large and small subunit

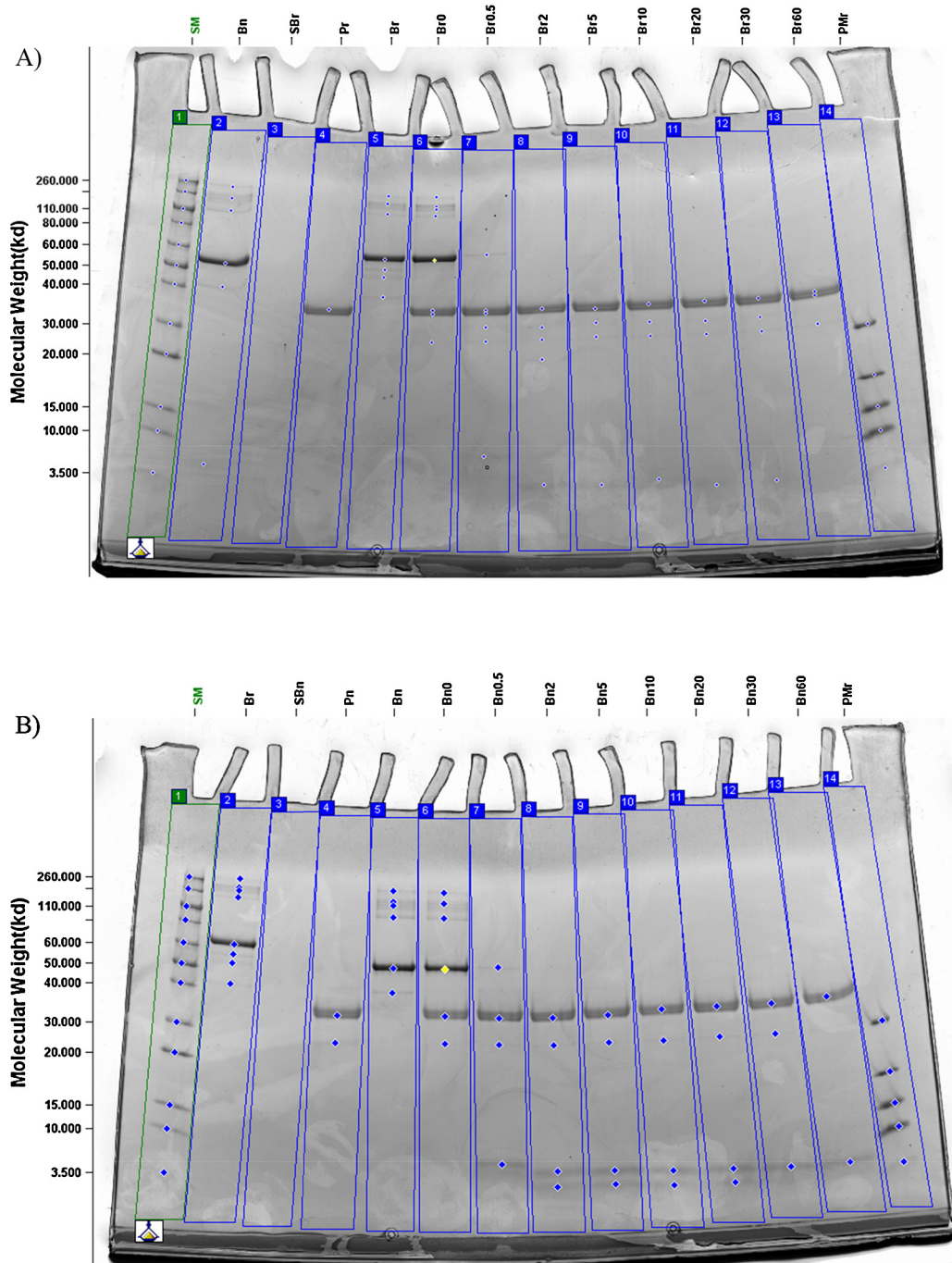
bands no longer visible by 30 s, a number of digestion fragments were observed in the 10–13 kDa region, persisting until 30 min. Under non-reduced conditions no significant bands were observed for the standard sample, however a



**Fig. 7.** Reduced (A) and non-reduced (B) analyses of BSA under Krypton staining conditions. From left to right for each gel the lanes contain: Novex sharp markers (Lane 1), test protein blank (opposite reduction status to digest samples) (Lane 2), sample buffer (Lane 3), pepsin blank (Lane 4), test protein blank (same reduction status as digest samples) (Lane 5), digest samples (Timepoints: T0, 0.5, 2, 5, 10, 20, 30, 60 min) (Lanes 6–13) and polypeptide SDS-PAGE standards (Lane 14).

stained region at the top of the lane was observed. Bands for a dimer of the large subunit at ~110 kDa (held by an intact disulphide bond under non-reducing conditions) and the small subunit were expected but not observed; it appears that when the disulphide bonds are not reduced the quaternary structure of the parent protein complex

remains stable under the analysis conditions. The intact parent complex is so large (~584 kDa) it cannot migrate under electrophoresis and so remains at the top of the lane. Analysis of the predicted digestion from Peptide Cutter suggested that a fragment consisting of two identical 16-mer peptides linked by a disulphide bond could be



**Fig. 8.** Reduced (A) and non-reduced (B) analyses of BSA under Coomassie Blue staining conditions. From left to right for each gel the lanes contain: Novex sharp markers (Lane 1), test protein blank (opposite reduction status to digest samples) (Lane 2), sample buffer (Lane 3), pepsin blank (Lane 4), test protein blank (same reduction status as digest samples) (Lane 5), digest samples (Timepoints: T0, 0.5, 2, 5, 10, 20, 30, 60 min) (Lanes 6–13) and polypeptide SDS-PAGE standards (Lane 14).

formed from the large subunit dimers, with a MW of  $\sim 3.5$  kDa, however this fragment was not observed under the conditions used. Fragments in the 8–10 kDa range were observed, and persisted until 30 min in a similar pattern to the reduced analysis. No significant bands remained by 60 min for either analysis condition; the apparent molecular weight of fragments observed under non-reduced

conditions differed slightly from that seen for the reduced analysis.

For BSA low molecular weight ( $<7$  kDa, LMW) fragments were observed under non-reduced conditions. As this protein has seventeen disulphide bonds, it is suggested these LMW fragments may have been composed of many very small fragments held together by the

disulphide bonds. The analysis of BSA digests was repeated using Coomassie Blue staining (Fig. 8), which gave better results on staining than the initial analysis using Krypton 1X staining. Detection of the LMW fragments in particular was enhanced using Coomassie Blue, with some fragments faintly visible under reducing conditions, and seen more clearly under non-reducing conditions. The fragments visible under reducing conditions were not as strongly stained as those reported by Thomas et al. [7]. As discussed in that paper, fixing and staining conditions can have a large impact on the detection of fragments in the lower MW ranges. The UniProt KnowledgeBase entry for BSA notes that the protein may be phosphorylated which may have contributed to the poor staining of the fragments in initial analysis with Krypton staining [16]. Analysis of the sequence using Peptide Cutter suggested that there are seven potential fragments composed of multiple shorter peptide sequences held by disulphide bonds, ranging from 15 to 43 amino acids (~1.6–4.7 kDa), which correspond to the observed fragments.

#### 4. Conclusions

Proteins with a range of disulphide bond structures were studied under reduced and non-reduced analysis in this study, and some notable differences in analysis results were observed. Furthermore, *in silico* prediction of digestion fragments is a useful complementary tool which may be used alongside disulphide bond information to design an appropriate approach to the *in vitro* assay, however the *in silico* results do not always reflect what is observed experimentally and should be considered a guide only. This is likely due to the accessibility of potential pepsin cleavage sites within the folded protein structure, as the Peptide Cutter tool only considers the linear sequence. In this paper, two proteins from the set of nine used by Thomas et al. [7] were shown to have significant differences between reduced and non-reduced analyses. In addition another protein from the set, the peanut allergen Ara h 2, has previously been shown to have significantly more resistance to pepsin digestion when analysed under non-reducing conditions [12]. The proteins were not selected for the original analysis based on disulphide bond arrangements, but as a representative set of allergens and non-allergens, and may not be considered an ideal set to study the effect of reduction on analysis. Despite this, significant differences have now been demonstrated for a third of the original set of proteins.

Careful consideration of protein structure is required when selecting SDS-PAGE sample conditions to assess the fragments resulting from *in vitro* pepsin digestion. Should the protein in question be known to have disulphide bonds present, it would be appropriate for researchers to consider whether a non-reduced element of analysis should be included in a modification of the widely accepted, and utilised, Thomas et al. [7] protocol. For proteins with disulphide bonds studied only under reducing conditions, significant fragments of digestion may be completely absent, and a full picture of the pepsin resistance would not be achieved. This could have a serious impact on the risk assessment of potential allergenicity.

Additionally, comparison of the results obtained with previously published studies showed some variability in the detection of fragments and persistence of parent proteins during the digestion time course. This may be attributable to experimental variation and the impact of different staining conditions, as the earlier reported work used solely Coomassie Blue staining, while the results presented herein were obtained with a fluorescent Krypton stain. Since the Thomas et al. [7] paper was published a plethora of new fluorescent stains have become available and the impact of the use of these in this assay is currently unclear and worthy of further exploration, such that recommendations can be standardised with regards to this aspect of the assay too. Where a difference in apparent MW is observed between reduced and non-reduced analyses, the use of a complementary technique to determine accurate MW for digestion fragments may be necessary, for example SEC-MALLS or LC-MS techniques.

In summary, the results reported here highlight that whilst providing a relatively simple, fast and cost-effective way of comparing a new protein of interest with known allergens and non-allergens, it should be recognised that one of the most widely accepted and used methods for assessing pepsin resistance [7] should not be considered fully standardised. Taking into consideration the protein(s) under assessment, there may be value in using different staining techniques and/or modifications such as use of non-reducing SDS-PAGE analysis.

As mentioned briefly in the introduction to this paper, there has been much interest and effort in recent years in developing approaches and models to more closely replicate human *in vivo* digestion, however the additional value these provide with regards to allergenicity risk assessment has not been systematically assessed. Indeed, as has been recently concluded as the result of a comprehensive literature review of this area [9], before a clear consensus can be reached as to what is meant by 'resistance' to digestion and the risk implications of such data, there is a pre-requisite need for standardised, harmonised test conditions, and fragment profiling, to enable comparison of test results for a range of reference proteins including allergens and non-allergenic controls. To add to the understanding of the impact of reducing or non-reducing conditions for such assays, and behaviour of proteins with multiple disulphide bonds, a further study is planned. An expanded set of proteins will be selected with a range of disulphide bond arrangements, in order to further demonstrate the importance of considering non-reduced analyses in experimental design.

#### Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

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