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ABSTRACT

The present study evaluated four laticifer fluids as a novel source of peptidases capable of hydrolyzing proteins in cow's milk. The latex peptidases from *Calotropis procera* (CpLP), *Cryptostegia grandiflora* (CgLP), and *Carica papaya* (CapLP) were able to perform total hydrolysis of caseins after 30 min at pH 6.5, as confirmed by a significant reduction in the residual antigenicity. Casein hydrolysis by *Plumeria rubra* latex peptidases (PrLP) was negligible. Moreover, whey proteins were more resistant to proteolysis by latex peptidases; however, heat pretreatment of the whey proteins enhanced the degree of hydrolysis and reduced the residual antigenicity of the hydrolysates. The *in vivo* assays show that the cow's milk proteins hydrolysed by CgLP and CapLP exhibited no immune reactions in mice allergic to cow's milk, similar to a commercial partially hydrolysed formula. Thus, these peptidases are promising enzymes for the development of novel hypoallergenic formulas for children with a milk allergy.

Keywords: Allergy; Laticifer; Protein; Protease.

1. Introduction

Peptidases are used in a wide range of industrial processes. Currently, the world market for these enzymes is in the order of billions of dollars annually (Singh, Mittal, Kumar & Mehta, 2016). The growing interest in these proteolytic enzymes is driven by their versatility, specificity, stability and high efficiency. Moreover, the use of peptidases represents an ecofriendly alternative to synthetic catalysts (Errasti, Caffini & López, 2018).

The hydrolytic action of peptidases has been applied to achieve chemical modifications to various foods, generating new products with improved sensory and nutritional quality, or even producing bioactive peptides (Giacometti & Buretic-Tomljanovic, 2017). Additionally, peptidases can also be used to reduce food allergenicity. Cow's milk proteins are among the primary cause of food allergy in infants and young children (Sicherer & Sampson, 2014). This immunological reaction can result in gastrointestinal, respiratory and dermatological problems (Fiocchi et al., 2010). The only approved therapy for food allergy is diet restriction, where a dairy substitute should be employed. Therefore, hypoallergenic cow's milk formulas are widely used (Souroullas, Aspri & Papademas, 2018), and extensively hydrolysed formulas or amino acid formulas are among the most effective alternatives employed in clinical practice. However, their usage is limited due to high costs (Fiocchi et al., 2018). Although peptidases from microbial, insect, plant and animal sources have been studied in the hydrolysis of cow's milk proteins, the search for novel proteolytic enzymes remains important, since some peptidases have exhibited technical drawbacks such as low yield and activity or very limited hydrolytic action towards milk proteins.

Latex is a milky plant fluid composed of a complex mixture of molecules, including proteolytic enzymes. The peptidase content of some latex samples can reach

90% of the total protein (Zare, Moosavi-Movahedi, Salami, Mirzaei, Saboury & Sheibani, 2013). This feature has enabled the use of these enzymes in different biotechnological approaches, such as the dehairing of leather (Lopéz et al., 2017) and milk clotting (Freitas et al., 2016). Accordingly, our hypothesis was that latex peptidases could be efficient molecules for the hydrolysis of proteins in cow's milk, producing hypoallergenic formulas. Therefore, the present work studied the proteolytic action of different latex peptidases on cow's milk proteins, as well as evaluating the *in vivo* allergencity of the hydrolysed proteins in a validated IgE-mediated food allergy mouse model.

2. Materials and methods

2.1. Reagents

Acrylamide (17-1302-02), bis-acrylamide (17-1304-02), sodium dodecyl sulphate (SDS) (17-1313-01), HiTrap Protein-A Sepharose high-performance column (17-0402-01), and Superdex 75 Increase 10/300 GL (29148721) were acquired from GE Healthcare Life Sciences (São Paulo, SP, Brazil). Azocasein (A2765), bromelain (code B4882), L-cysteine (C7352), O-phthaldialdehyde (P0657), β-mercaptoethanol (M6250), Evans blue dye (E2129), cholera toxin from *Vibrio cholerae* (C8052), Freund's complete (F5881) and incomplete (F5506) adjuvant, alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (A6066) and *p*-nitrophenylphosphate disodium (N2765) were obtained from Sigma-Aldrich (São Paulo, SP, Brazil). All other chemicals were of analytical grade.

2.2. Plant material and proteolytic activity

Latex proteins (LP) from *Calotropis procera* (CpLP), *Cryptostegia grandiflora* (CgLP), *and Plumeria rubra* (PrLP) were collected by cutting the end branches of each

plant, as described by Freitas et al. (2007; 2010), whereas *Carica papaya* (CapLP) latex proteins were obtained from the green fruits, as reported by Souza et al. (2011). The latex fluids were collected in distilled water (1:1 ratio), and the rubber was separated by centrifugation (10,000 x g at 4 °C for 10 min). The supernatants were dialysed against distilled water for two days at 4 °C using membranes with an 8-kDa cut-off, followed by lyophilisation and storage until further analysis. The total proteolytic activity of all latex protein (LP) fractions was determined using 1% azocasein at pH 6.5 (milk pH) as a non-specific substrate prior to the performance of hydrolysis assays to ensure that the latex peptidases were active (Freitas et al., 2007). The quality controls for all samples in terms of protein profile and enzymatic performance, including autolysis assays, were evaluated by SDS-PAGE as described previously (Freitas et al., 2007; 2010; 2016). For all experiments, bromelain (EC 3.4.22.32), a cysteine peptidase from pineapple stem, was used as the positive control. The protein content of all latex samples was determined according to the Bradford procedure, with bovine serum albumin as the protein standard (Bradford, 1976).

2.3. Purification of cow's milk proteins

Caseins and whey proteins were purified as described by Oliveira et al. (2018). Briefly, whole bovine milk (Itambé®, Brazil) obtained from a local market (Fortaleza, Ceará, Brazil) was skimmed by centrifugation (2,100 x g at 25 °C for 30 min), and the supernatant was acidified with 1 M HCl to pH 4.6. Subsequently, the caseins and whey proteins were separated by centrifugation (1,500 x g at 20 °C for 20 min); the supernatant (whey proteins) was collected and separated, and the precipitate (sodium caseinate) was washed three times with distilled water and centrifuged. Both fractions were dialysed

against distilled water at 4 °C for 48 h using dialysis membranes with an 8-kDa cut-off and then lyophilized.

2.4. Hydrolysis of milk proteins

2.4.1. Casein hydrolysis

The hydrolysis of caseins was performed by incubating different sized aliquots (10, 15, 20, 25, and 30 µl) of each LP fraction (2 mg/ml in 50 mM Tris-HCl buffer pH 6.5 containing 1 mM L-cysteine) with 450 µl caseins (10 mg/ml in Tris-HCl buffer pH 6.5), with the final volume adjusted to 500 µl using the same buffer. The reactions were performed at 37 °C for 30 min, and 5 µl aliquots were retrieved for measurement of the extent of hydrolysis by 15% SDS-PAGE (Oliveira et al., 2018). The degree of hydrolysis was also measured using O-phthaldialdehyde (OPA) reagent according to Church, Swaisgood, Porter, and Catignani (1983). OPA reagent was prepared as follows: 25 ml 100 mM sodium tetraborate; 2.5 ml 20% SDS; 40 mg OPA dissolved in 1 ml methanol; 100 µl β-mercaptoethanol, adjusted to a final volume of 50 ml with distilled water. Aliquots of 50 µl casein hydrolysates were mixed with 1 ml OPA reagent, and the absorbance was measured after 2 min at 340 nm (Church et al., 1983).

2.4.2. Whey and whole cow's milk protein hydrolysis

Since whey proteins have been reported to be more resistant to proteolysis, assays were performed using the highest concentration of LP for different incubation times. A volume of 30 µl each LP fraction (2 mg/ml in Tris-HCl buffer pH 6.5 containing 1 mM L-cysteine) was mixed with 450 µl whey proteins (10 mg/ml in 50 mM Tris-HCl buffer

pH 6.5) or whole cow's milk (Integral milk powder, Itambé®, Brazil), adjusted to a final volume of 500 µl using the same buffer. The reactions were performed at 37 °C, and the extent of hydrolysis was monitored at different time points (1, 2, 4, and 24 h) by 15% SDS-PAGE and/or size exclusion chromatography.

For SDS-PAGE analysis, 5 µl aliquots of the hydrolysates were mixed with sample buffer (1:1, v:v) (0.0625 M Tris buffer (pH 6.8) containing 2% SDS). Electrophoresis was performed at 25 mA and 25 ºC for 2 h, followed by the staining of gels with Coomassie Brilliant Blue (R-350) solution in water:acetic acid:methanol (7:1:2, v:v:v) and de-colouration with the same solution without the dye (Oliveira et al., 2018).

For chromatographic assays, the whey protein hydrolysates (500 µl) were loaded into a Superdex-75 (10/300 GL) column, previously equilibrated with 50 mM Tris-HCl buffer pH 6.5, coupled to a high-performance liquid chromatographic system (AKTA purifier, GE Healthcare). Proteins were eluted with 50 mM Tris-HCl buffer (pH 6.5) at a 0.3 ml/min flow rate, and the peaks were monitored at 280 nm.

To improve the degree of hydrolysis of whey proteins by latex peptidases, the whey proteins or whole cow's milk (Integral milk powder, Itambé®, Brazil) (10 mg/ml in 50 mM Tris-HCl buffer pH 6.5) were preheated at 85 °C for 30 min prior to incubation with each latex fraction for 24 h, as described earlier. Hydrolysis yield was analysed by 15% SDS-PAGE and an enzyme-linked immunosorbent assay (ELISA).

2.5. Polyclonal antibody production and ELISA

Rabbit polyclonal antibodies against bovine caseins and whey proteins were produced according to Oliveira et al., (2018). Briefly, the animals were sensitised intramuscularly either with caseins or whey proteins (1 mg dissolved in 0.5 ml saline and

0.5 ml complete Freund's adjuvant), and booster injections (1 mg caseins or whey proteins dissolved in 0.5 ml saline and 0.5 ml incomplete Freund's adjuvant) were administered subcutaneously after 21, 35 and 42 days. The immunoglobulins were purified using Protein A immobilized on a Sepharose 4B column, as described by Freitas et al. (2017).

The residual antigenicity of the casein, whey protein (unheated and preheated at 85 °C for 30 min), and whole cow's milk protein (preheated at 85 °C for 30 min) hydrolysates was measured using ELISA, according to Oliveira et al. (2018). Aliquots (150 µl) of the same samples used in the *in vitro* assays (sections 2.4.1 and 2.4.2) were added to 96-well microplates and stored overnight at 4 °C. Unbound proteins were removed by washing, and the empty sites were blocked with gelatin (150 µl, 10 mg/ml). Anti-casein and anti-whey protein polyclonal antibodies (150 µl, 1:20,000 dilution) were applied, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (150 µl, 1:10,000 dilution) as the secondary antibody. The reaction was detected using *p*-nitrophenyl phosphate disodium (150 µl, 5 mg/ml) as the substrate, and the absorbance was measured at 405 nm. Enfamil® Gentlease® formula (Mead Johnson Nutrition) (150 µl, 10 mg/ml) was used as the control for partially hydrolysed cow's milk (PHM) and whole cow's milk (Integral milk powder, Itambé®, Brazil) as non-hydrolysed milk (NHM) (150 µl, 10 mg/ml).

2.6. Mice sensitisation and challenge

Six- to eight-week-old male mice were purchased from the School of Animal Sciences, University of La Plata (UNLP), Argentina, and kept under pathogen-free conditions at 20 ºC, 70% relative humidity, and a 12/12 h light/dark cycle, with water and

commercial diet provided *ad libitum*. The sensitisation protocol was approved by the Institutional Committee for the Care and Use of Laboratory Animals at the School of Sciences UNLP (CICUAL-FCE, Argentina) (protocol #017-10-15) and performed according to Candreva, Smaldini, Curciarello, Fossati, Docena and Petruccelli (2016). Briefly, mice were divided into sensitised (n = 20) and control (n = 5) groups. The sensitised group received six weekly intragastric doses of whole cow's milk (Integral milk powder, Itambé®, Brazil) (20 mg/dose) with cholera toxin (10 µg/dose) dissolved in 200 µl 125 mM bicarbonate buffer. The control group received only whole cow's milk (20 mg/dose in 200 µl). Ten days after the final booster, sensitised mice were intragastrically challenged with 20 mg whole cow's milk (NHM) or whole cow's milk hydrolysed by different latex peptidases (CpLP, CgLP, and CapLP). For hydrolysis, 60 µg each LP was incubated with 4.5 mg whole cow's milk (Integral milk powder, Itambé®, Brazil) that had been preheated for 30 min at 85 ºC. The reactions were performed for 24 h at 37 ºC, and the materials were subsequently lyophilised and used in the *in vivo* assays (20 mg/dose). Enfamil® Gentlease® formula (Mead Johnson Nutrition) (20 mg/dose) was used as the control for partially hydrolysed cow's milk (PHM) and whole cow's milk (20 mg/dose) as non-hydrolysed milk (NHM). These same samples were also evaluated by SDS-PAGE and ELISA, as described earlier. Sensitisation was controlled by measuring milk-specific IgE antibodies in serum by ELISA, as described by Smaldini et al. (2012).

2.7. In vivo evaluation of allergic reactions

Symptoms were evaluated in sensitised and control mice following oral challenge and scored according to Table 1. The mice were observed 30 min after the oral challenge in a blinded fashion, and two independent investigators assigned the scores. Cutaneous tests were performed according to Candreva et al. (2016).

Mice in the sensitised and control groups received subcutaneous injection of the non-hydrolysed cow's milk (NHM), partially hydrolysed cow's milk (PHM), or whole cow's milk hydrolysed by CpLP, CgLP, or CapLP in the footpad (1 µg/µl or 20 µg in sterile saline), followed by an intravenous injection of 100 µl 0.1% Evans blue dye. Saline buffer was injected in all animals in the contralateral footpad as a control. Blue colour observed in the skin pad a few minutes after the injection was considered a positive cutaneous test. The footpad swelling was quantitated using a digital micrometer with a minimum increment of 0.01 mm.

2.8. Statistical analysis

The data are expressed as the mean ± SD of three independent assays. Statistical analyses were performed by the GraphPad Prism 5 software using ANOVA followed by multiple comparison using the Student–Newman–Keuls test. In all tests, $p < 0.05$ is considered statistically significant.

3. Results

3.1. Hydrolysis of milk proteins by latex peptidases

Figure 1 shows the hydrolytic potential of different latex peptidases against bovine caseins. A differential hydrolysis of bovine caseins during 30 min was found according to the peptidase employed. As seen in Fig. 1a, the peptidases from *C. procera* (CpLP), *C. grandiflora* (CgLP), and *Carica papaya* (CapLP) latex extensively processed the caseins, even at the lowest concentration assessed (20 µg LP per 4.5 mg casein; 1:225 ratio). Similar results were observed for bromelain, a peptidase used as the positive control. On the contrary, *P. rubra* (PrLP) latex peptidases were unable to hydrolyse caseins even at the highest concentration (60 µg LP per 4.5 mg casein; 1:75 ratio). The degree of casein

proteolysis by different latex peptidases was also quantitated by the OPA method (Fig. 1b). We observed concentration-dependent hydrolysis for CpLP, CgLP, CapLP, and bromelain. The maximum hydrolysis was reached using 60 µg each fraction. CapLP exhibited the highest degree of casein hydrolysis, followed by bromelain, CgLP, and CpLP. In concordance with the results depicted in Fig. 1a, PrLP showed no significant proteolysis, confirming its inability to hydrolyse bovine caseins (Fig. 1b).

A similar analysis was carried out with whey proteins, and it was found that this milk fraction was resistant to proteolysis using latex peptidases, even at the highest concentration (60 µg LP per 4.5 mg whey protein; 1:75 ratio). SDS-PAGE patterns showed that only CapLP exhibited substantial hydrolysis of β-lactoglobulin (β-LG) and α-lactoalbumin (α-LA). Hydrolysis could be observed after a 2 h treatment and proceeded to almost complete hydrolysis by 24 h (Fig. 2). These findings were confirmed by size exclusion chromatography. We observed a reduction in the eluted components of the chromatographic peaks corresponding to β-lactoglobulin (β-LG) and α-lactoalbumin (α-LA) hydrolysed by CapLP (around 90%). The other peptidases showed low hydrolysis of whey proteins after a 24 h incubation. These results were similar to those observed with bromelain, which were better monitored by chromatography analysis than by SDS-PAGE. PrLP was unable to perform any hydrolysis of whey proteins (Fig. 2).

3.2. In vitro residual antigenicity

The *in vitro* residual antigenicities of the hydrolysed caseins and whey proteins were evaluated by ELISA using anti-casein and anti-whey protein polyclonal antibodies, respectively (Fig. 3a and 3b). The immunological recognition of the remaining casein peptides following a 30 min treatment was significantly reduced when CpLP (2%), CgLP (1%), CapLP (2%), and bromelain (1%) were used (1:75 ratio) as compared with

untreated caseins (100%). Treatments resulted in a higher degree of hydrolysis as compared with the commercial partial hydrolysate employed as the control (PHM) ($p < 0.05$). In contrast, we found a high residual antigenicity when caseins were treated with PrLP (Fig. 3a).

Whey proteins were assessed using the same method, and the residual antigenicity following a 24 h incubation (1:75 ratio, enzyme:substrate) was partially reduced. We observed a significant reduction in antigenicity with CpLP, CgLP, and bromelain as compared with untreated whey proteins, similar to the commercial partially hydrolysed milk formula (PHM). The residual antigenicity values after a 24 h proteolysis were 78% for CpLP, 71% for CgLP, 31% for CapLP, 62% for bromelain, and 50% for PHM, as compared with non-hydrolysed whey proteins (100%) ($p < 0.05$). This assay again showed that PrLP did not reduce whey protein antigenicity (Fig. 3b) ($p > 0.05$).

3.3. Analysis of the hydrolysis of heated whey proteins

Whey proteins were pretreated (85 °C for 30 min) to enhance proteolysis by latex peptidases (Fig. 4). The hydrolysis of β-lactoglobulin (β-LG) and α-lactoalbumin (α-LA) by CapLP and bromelain was greatly enhanced as compared with the results obtained with the unheated proteins (Fig. 2). SDS-PAGE showed a large number of peptides with reduced relative molecular weights (Fig 4a) and significantly reduced antigenicity when the samples were treated with CpLP, CgLP, and bromelain (Fig. 4b). PrLP did not cleave preheated whey proteins, as showed by SDS-PAGE. ELISA data were consistent with SDS-PAGE patterns, suggesting that pretreatment (85 °C for 30 min) enhanced the hydrolysis of whey proteins by CapLP, CpLP, and CgLP.

3.4. In vivo residual allergenicity

The viability of different latex peptidases as enzymatic sources for the hydrolysis of whole cow's milk proteins (preheated at 85 °C for 30 min) was also assessed by SDS-PAGE, ELISA and a milk-specific food allergy mouse model. Milk-specific IgE production was assessed by ELISA on days 0 and 20 and following oral challenge (data not shown). Allergenicity of the residual hydrolysates was evaluated by oral challenge and skin testing in sensitised and control mice. Samples assessed were those of whole milk proteins hydrolysed with peptidases that cleaved milk fractions (*in vitro* analysis). Clinical scores of the challenged mice were calculated according to the symptoms elicited immediately following gavage, according to Table 1.

To confirm the hydrolytic potential of latex peptidases (CpLP, CgLP, and CapLP), whole cow's milk (Integral milk powder, Itambé®, Brazil), preheated at 85 °C for 30 min, was used as the substrate, and the resulting hydrolysis was compared with non-hydrolysed (Itambé®) and commercially available partially hydrolysed (Nan Supreme® and Enfamil®) milks by SDS and ELISA (Supplementary Fig. 1). Following a 24 h incubation at 37 °C, the hydrolytic patterns and residual allergenicity of caseins and whey proteins after hydrolysis by latex peptidases were very similar to those of the purified milk proteins (Figs. 1 and 4), reinforcing the potential of latex peptidases, since the presence of fats, carbohydrates, and salts did not decrease their enzymatic action.

As observed in Fig. 5a, oral challenge with whole cow's milk proteins hydrolysed by CgLP and CapLP elicited no immune response (clinical scores) in sensitised animals as compared with non-hydrolysed milk (NHM). The results were similar to commercially available partially hydrolysed milk formula (PHM). Control animals showed no symptoms following all challenges (PBS). Whole milk proteins treated with CpLP showed intermediate clinical scores as compared with NHM ($p < 0.05$). The animals

displayed scratching and rubbing around the snout and head (score 1), puffiness around the eyes and mouth, piloerection, reduced activity, and/or decreased activity with increased respiratory rate (score 2), or no activity upon stimuli and convulsion (score 4). As controls, sensitised mice received NHM, PHM, or PBS by gavage, and clinical scores of 4, 0, and 0 were observed, respectively.

Quantitation of foot pad swelling showed a significant reduction in inflammation when the skin test was performed with all hydrolysates as compared with NHM (Fig. 5b). No critical adverse effects were observed with any of the assessed hydrolysates. Finally, the skin test results are consistent with the previous findings. An intense blue colour was observed when sensitised animals were injected with non-hydrolysed cow's milk proteins (NHM), representing the highest degree of inflammation. Cow's milk proteins hydrolysed by CgLP and CapLP exhibited results similar to those seen with partially hydrolysed milk formula (Enfamil®) (PHM) (Fig. 5c). All sensitised mice injected in the contralateral footpad with PBS showed a negative skin test. Overall, the *in vivo* results suggest that milk proteins treated with CpLP and CapLP had the lowest reaction in allergic mice.

4. Discussion

Enzymatic hydrolysis is the most promising strategy for decreasing the antigenicity and allergenicity of cow's milk proteins. This enzymatic procedure disrupts lineal and conformational epitopes, preventing IgE-mediated responses (Bu, Luo, Chen, Liu & Zhu, 2013). The extent of protein hydrolysis by peptidases is a critical issue in the development of hypoallergenic milk formulas for allergic children. Although some plant peptidases, such as bromelain and ficin, have been highlighted in this process (Abd El-Salan & El-Shibiny, 2017), the search for novel plant proteolytic enzymes remains the

focus of numerous studies and patents. In the present study, latex from four different species (*C. procera*, *C. grandiflora*, *C. papaya*, and *P. rubra*) was evaluated as a potential enzymatic source for the hydrolysis of cow's milk proteins, since previous studies have reported their proteolytic potential (Freitas et al., 2007; 2010). The *C. procera*, *C. grandiflora*, and *C. papaya* lattices are rich in cysteine peptidases, while the *P. rubra* latex contains a mixture of cysteine and serine peptidases (Freitas et al., 2010). On the other hand, bromelain (a cysteine peptidase from pineapple) was used as the positive control, since previous studies have reported its capacity to hydrolyse cow's milk proteins (Medeiros, Rainha, Paiva, Lima & Baptista, 2014).

The extent of protein hydrolysis can be affected by peptidase specificity, as well as by the hydrolysis conditions such as pH, temperature, ions, enzyme:substrate ratio, and reaction time (Abd El-Salan & El-Shibiny, 2017). In contrast to some standard animal peptidases such as trypsin, chymotrypsin, and pepsin, the plant cysteine peptidases are able to hydrolyse proteins at several cleavage sites, promoting extensive proteolysis (Hedstrom, 2002; Choe et al., 2006). Therefore, from this standpoint, plant cysteine peptidases exhibit higher biotechnological potential for the production of hypoallergenic milk formulas or other kinds of hydrolysed foods. In hydrolysis reactions, the pH should be close to the optimum pH value of the enzyme in order to reach the maximum cleavage of the substrate. Previous studies have reported that *C. procera*, *C. grandiflora*, *C. papaya*, and *P. rubra* peptidases have optimal enzymatic activities at pH values close to 6.5 (milk pH) (Freitas et al., 2007; 2010). Thus, proteolysis performed using latex peptidases has this advantage as compared with serine peptidases, which are more active at alkaline pH values such as 9.0–10.0 (Oliveira et al., 2018). The presence of certain ions can also be an important element in enzymatic reactions, since they can decrease the activity, or even inactivate enzymes. The calcium ions, abundant in milk, did not affect

the proteolytic activity of *C. procera*, *C. grandiflora*, and *C. papaya*, even at 1 M $CaCl_2$ (Freitas et al., 2016). Time can also limit enzymatic reactions catalysed by peptidases, since these enzymes can be inactivated by autodigestion (Oliveira et al., 2018). Interestingly, *C. procera* and *C. grandiflora* latex peptidases did not undergo autolysis and were active even after a 24 h incubation at 37 ºC (Freitas et al., 2016). All these biochemical characteristics strengthen the evidence that peptidases from *C. procera*, *C. grandiflora*, and *C. papaya* present high stabilities and have potential uses in the food industry.

Here, we show that latex peptidases from *C. procera*, *C. grandiflora*, and *C. papaya* hydrolysed bovine caseins within 30 min, similar to bromelain. The residual antigenicity data confirmed that caseins were extensively hydrolysed by these latex peptidases, since anti-casein polyclonal antibodies barely detected the hydrolysed peptides. Furthermore, casein hydrolysates exhibited less immunoreactive peptides as compared with a commercially available hypoallergenic formula (Enfamil®). Comparison of different peptidases showed that papain (peptidase from *C. papaya* latex) was more efficient than trypsin and pancreatin in the hydrolysis of bovine caseins (Luo, Pan, & Zhong, 2014). In another study, anti-casein polyclonal antibodies were used to detect the residual antigenicity of different hydrolysed formulas, and casein components were detected even in extensively hydrolysed formulas (Plebani et al., 1997). Since caseins are the most abundant cow's milk proteins (about 80%) and also comprise the major antigenic proteins in bovine milk (Docena, Fernandez, Chirdo & Fossati, 1996), their extensive hydrolysis is essential in the production of hypoallergenic formulas.

In contrast to caseins, whey proteins were much more resistant to proteolysis by latex peptidases. The resistance of both α-lactalbumin and β-lactoglobulin reflects their intrinsic structural features and compact structures, which are stabilised by disulphide

bonds (Papiz et al., 1986; Permyakov & Berliner, 2000). Whey protein hydrolysis by latex peptidases was only partial, and residual antigenicity was detected in the hydrolysates, although to a lesser extent than in the commercially available hypoallergenic formula (Enfamil®). Similar results were reported by Quintieri, Monaci, Baruzzi, Giuffrida, Candia and Caputo (2017); using SDS-PAGE, the authors showed that papain hydrolysed whey proteins, but residual antigenicity was detected by ELISA. Other peptidases have been studied with respect to the hydrolysis of whey proteins without success (Cheison, Leeb, Toro-Sierra & Kulozik, 2011). Therefore, attempts to improve the degree of hydrolysis of whey proteins are focussed on changing the hydrolysis conditions and/or pretreatment of the substrate (Cheison & Kulozik, 2017). Among different strategies, heat denaturation has been investigated with the aim of improving hydrolysis and consequently decreasing their residual antigenicity (Oliveira et al., 2018). Certain whey proteins are thermolabile; thus, heating can cause structural alterations that facilitate proteolysis (Reddy, Kella & Kinsella, 1988). In the present study, the degree of hydrolysis by latex peptidases increased following heat denaturation of whey proteins, with a subsequent reduction in the residual antigenicity. Similarly, heat treatment slightly enhanced the peptic and tryptic hydrolysis of whey proteins (Adjonu, Doran, Torley, & Agboola, 2013). In addition, Kim and collaborators (2007) showed that the reduction in the residual antigenicity of whey proteins was also greater when hydrolysis was performed with two enzymes. This result reinforces the potential of latex fluids, since they are rich in multiple peptidases. More than 100 different lattices are known to contain at least one peptidase (Domsalla & Melzig, 2008). In addition to papain, the latex from *C. papaya* (CapLP) contains three other cysteine peptidases (Mezhlumyan, Kasymova, & Yuldashev, 2003). Similarly, the latex from *C. procera* (CpLP), *C. grandiflora* (CgLP), and *P. rubra* (PrLP) possesses multiple cysteine peptidases (Freitas et al., 2007; 2010).

The allergenicity of cow's milk hydrolysates was also studied using a milk-specific IgE-mediated food allergy mouse model. Studies using animal models are essential as a biological tool for the evaluation of the hypoallergenicity of processed foods or proteins. Previous validation of this mouse model showed that casein- and whey-specific IgE antibodies are elicited, although casein is the major allergen in milk. The existence of remaining IgE epitopes that could crosslink IgE molecules on the surface of mast cells and basophils, and hence trigger hypersensitivity reactions immediately following the challenge, was evaluated. This is the only way to characterise the sensitising capacity and residual immunological response of hydrolysed formulas (Fritsché, 2003). Here, we show that CpLP hydrolysates triggered hypersensitivity symptoms and cutaneous reactions in sensitised mice, likely caused by the high residual antigenicity of whey proteins. Interestingly, cow's milk hydrolysed by CgLP and CapLP generated weak immune responses and no symptoms, similar to the commercially available formula, Enfamil®. There are several definitions of hypoallergenic formulas, but the most stringent states that they must be tolerated by 90% of allergic infants, without eliciting any symptoms (Kleinman, Bahna, Powell & Sampson, 1991). Some studies have shown that several IgE epitopes can still remain intact following proteolysis. Based on this, even extensively hydrolysed formulas can elicit immune reactions (Docena, Rozenfeld, Fernández & Fossati, 2002). Therefore, the implementation of complementary techniques, such as ultrafiltration is recommended to eliminate antigenic peptides containing potential B epitopes, and even intact proteins (Quintieri, Monaci, Baruzzi, Giuffrida, Candia & Caputo, 2017).

5. Conclusion

C. grandiflora (CgLP) and *C. papaya* (CapLP) latex peptidases were found to be the best enzymes for the hydrolysis of milk proteins, exhibiting the best performance in different *in vitro* and *in vivo* tests in terms of reduced antigenicity and allergenicity. Thus, these peptidases are promising enzymes for the development of novel hypoallergenic formulas for children with a milk allergy, confirming our initial hypothesis.

Conflict of interest

The authors confirm that the contents of this article pose no conflicts of interest.

Contributions

JPBO, MVR and CDTF performed the main research work, including latex peptidase purification, gel electrophoresis, proteolytic activity assays and hydrolysis assays. MBA, JSO and HDO produced polyclonal antibodies and performed ELISA. AMC and GR performed the *in vivo* assays, while GD supported and designed *in vivo* studies. All authors contributed to data analysis, discussion and writing of the manuscript.

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Figure legends:

Fig. 1. Bovine casein hydrolysis by different latex peptidases evaluated by 15% SDS-PAGE (a) and a colourimetric assay (b). Legend: C (Control), non-hydrolysed caseins; CNs, total casein fraction; and CpLP, CgLP, CapLP, and PrLP, latex peptidases from *C. procera*, *C. grandiflora*, *C. papaya*, and *P. rubra*, respectively. Bromelain, a cysteine peptidase, was used as the positive control. In "b", the degree of hydrolysis was evaluated using the O-phthaldialdehyde (OPA) method. Each value represents the mean of three independent experiments ± SD. The hydrolysis assays were performed at 37 °C (pH 6.5) for 30 min using different concentrations of LP (20, 30, 40, 50, and 60 μg protein) per 4,500 μg total casein fraction, corresponding to 1:225, 1:150, 1:112.5, 1:90, and 1:75 ratios, respectively.

Fig. 2. Bovine whey protein hydrolysis by latex peptidases measured by 15% SDS-PAGE and size exclusion chromatography. Legend: C (Control), non-hydrolysed whey proteins; CpLP, CgLP, CapLP, and PrLP, latex peptidases from *C. procera*, *C. grandiflora*, *C.*

papaya, and *P. rubra*, respectively; β-LG, β-lactoglobulin; α-LA, α-lactoalbumin. Bromelain, a cysteine peptidase, was used as the positive control. Assays were performed at 37 °C (pH 6.5) for different time periods (1, 2, 4, and 24 h) and at an enzyme:substrate ratio of 1:75, corresponding to 60 μg LP:4,500 μg whey protein.

Fig. 3. Residual antigenicity of cow's milk measured by ELISA using polyclonal antibodies against caseins and whey proteins. (a) The casein fraction was hydrolysed for 30 min at an enzyme:substrate ratio of 1:75 (60 μg enzyme:4,500 μg caseins) at 37 ºC. (b) Whey proteins were hydrolysed for 24 h at an enzyme:substrate ratio of 1:75. Each value represents the mean ± SD. Different letters represent significant differences ($p < 0.05$) between the indicated group and the control. Legend: CpLP, CgLP, CapLP, and PrLP, milk proteins hydrolysed by latex peptidases from *C. procera*, *C. grandiflora*, *C. papaya*, and *P. rubra*, respectively. Bromelain, a cysteine peptidase, was used as the positive control. PHM, Enfamil® Gentlease® formula (Mead Johnson Nutrition), was used as the control for partially hydrolysed cow's milk.

Fig. 4. Effect of preheating on the degree of hydrolysis of whey proteins measured by 15% SDS-PAGE (a) and ELISA (b). Whey proteins were preheated at 85 ºC for 30 min prior to enzyme addition. Assays were performed at 37 °C (pH 6.5) for 24 h at an enzyme:substrate ratio of 1:75. Densitometry of the protein bands in (a) was measured using the IMAGEJ software. Each value represents the mean ± SD. Different letters represent significant differences ($p < 0.05$) between the indicated group and the control. Legend: C (Control), non-hydrolysed preheated whey proteins; Preheated whey proteins incubated with latex peptidases from *C. papaya* (CapLP), *C. grandiflora* (CgLP), *C. procera* (CpLP), *P. rubra* (PrLP), and (b) bromelain; β-LG, β-lactoglobulin; α-LA, α-lactoalbumin.

Fig. 5. *In vivo* responses of allergic mice following administration of cow's milk hydrolysed by latex peptidases. (a) Clinical scores corresponded to the symptoms observed 30 min following oral challenge with milk proteins hydrolysed by latex peptidases. The scores were assigned according to Table 1. (b) Increase in the footpad thickness and (c) Cutaneous test following footpad injection of milk proteins hydrolysed by latex peptidases. Blue colour observed in the skin after the injection was considered a

positive cutaneous reaction. Data are expressed as the mean ± SD. Statistically significant difference by ANOVA: ***$p < 0.01$, *$p < 0.05$. Legend: NHM, non-hydrolysed milk; PHM, partially hydrolysed milk; PBS, phosphate buffered-saline; milk proteins hydrolysed by latex peptidases from *C. procera*, *C. grandiflora*, and *C. papaya*; CpLP, CgLP, and CapLP, respectively.

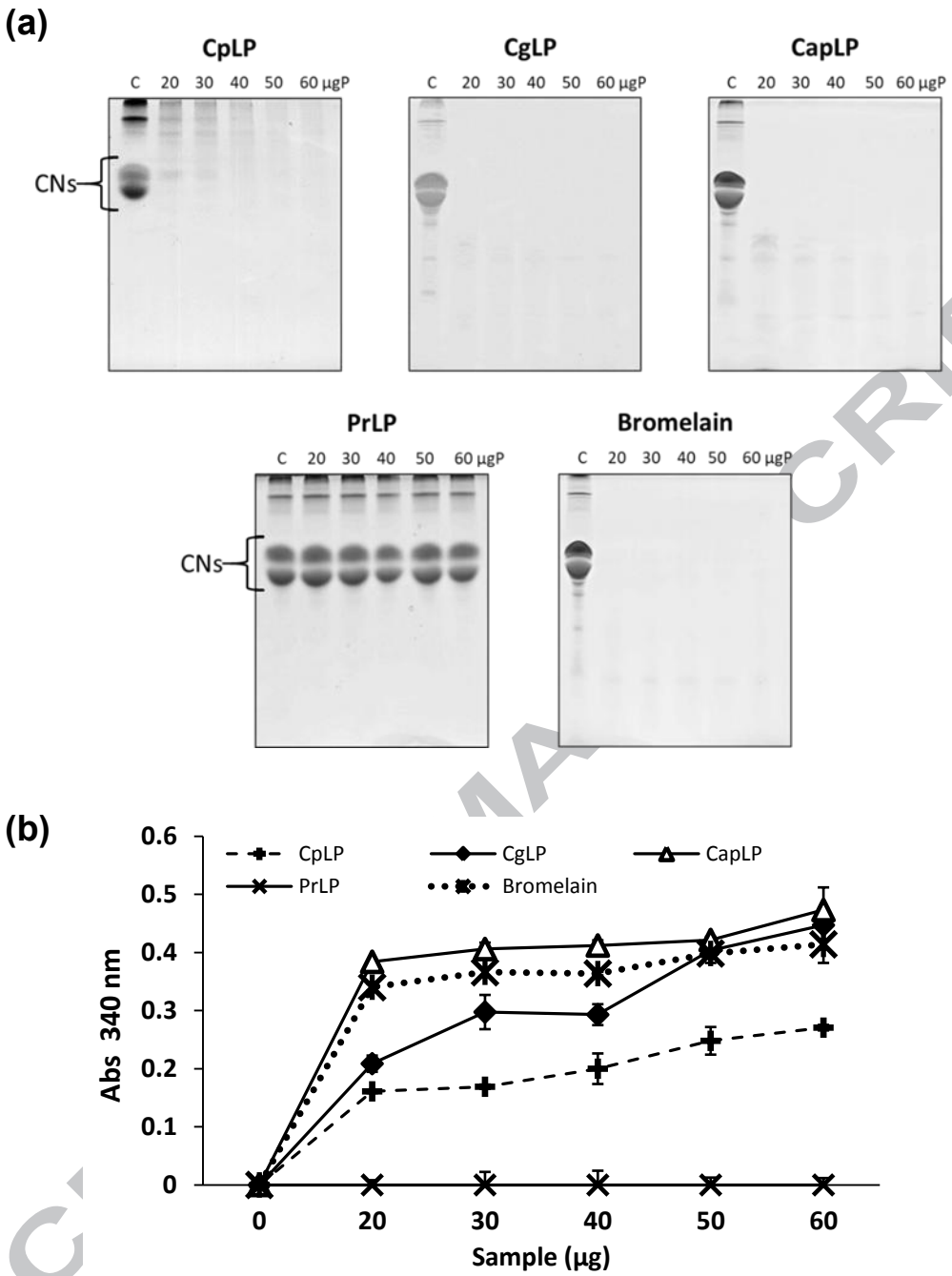


Fig.1.

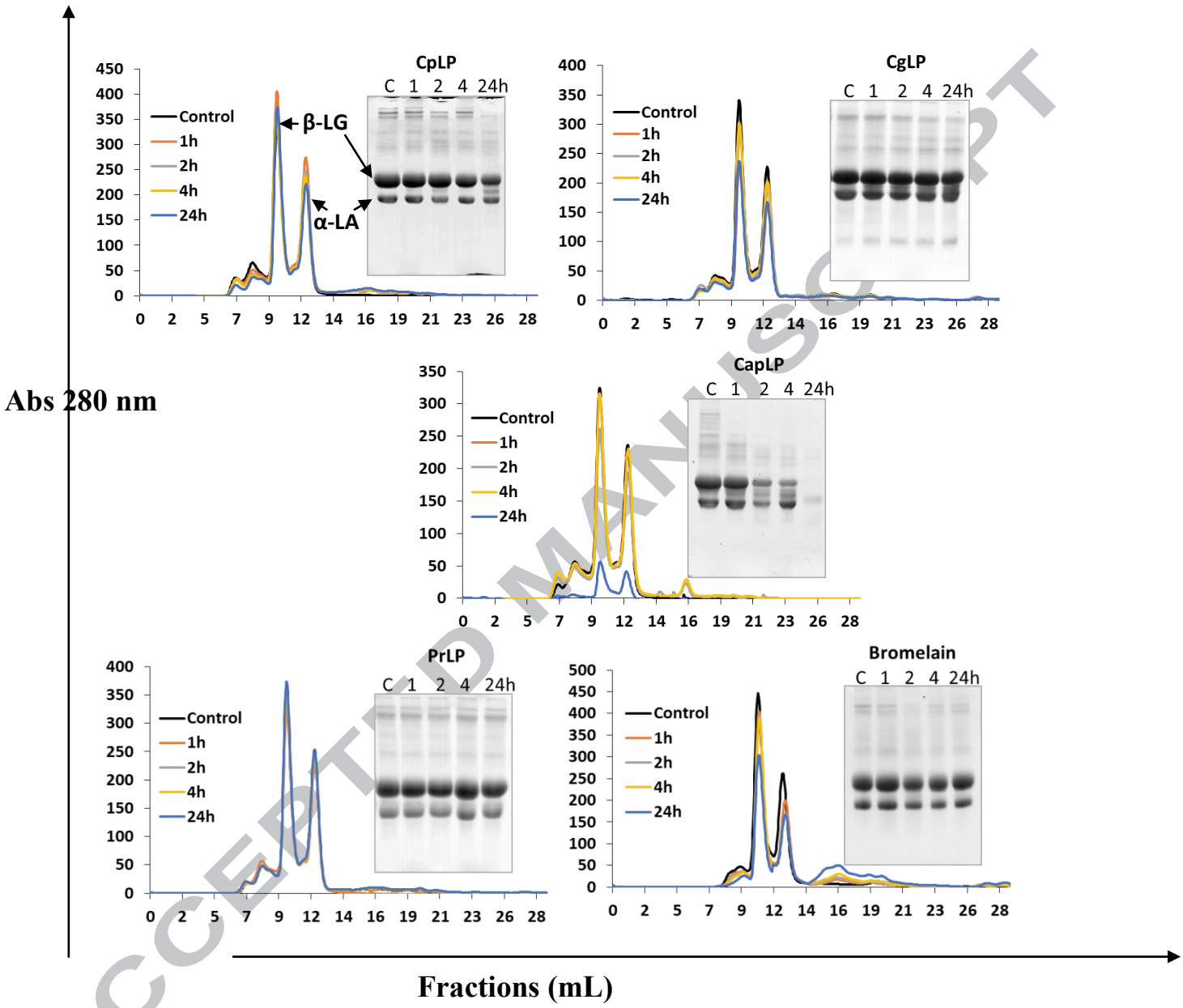
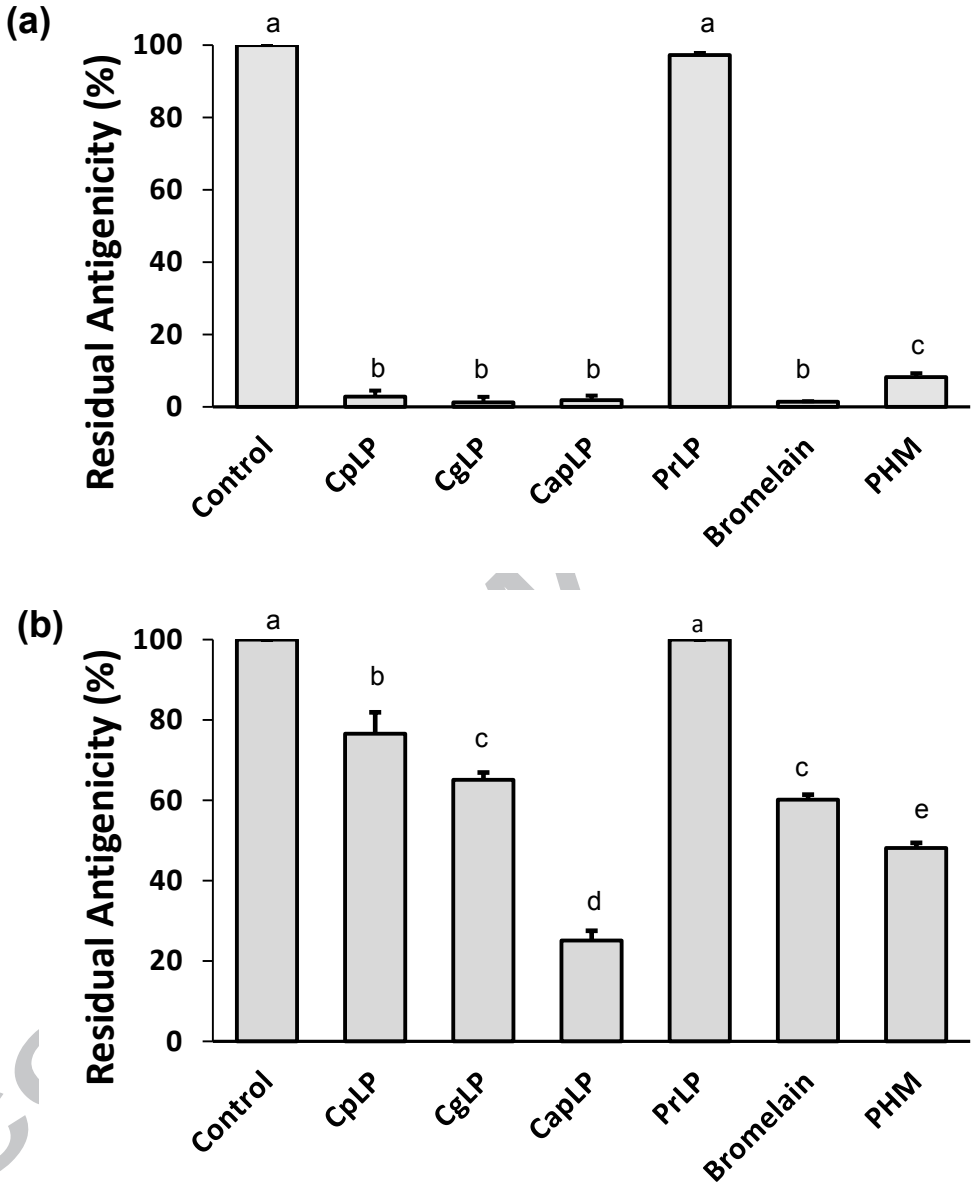


Fig.2.



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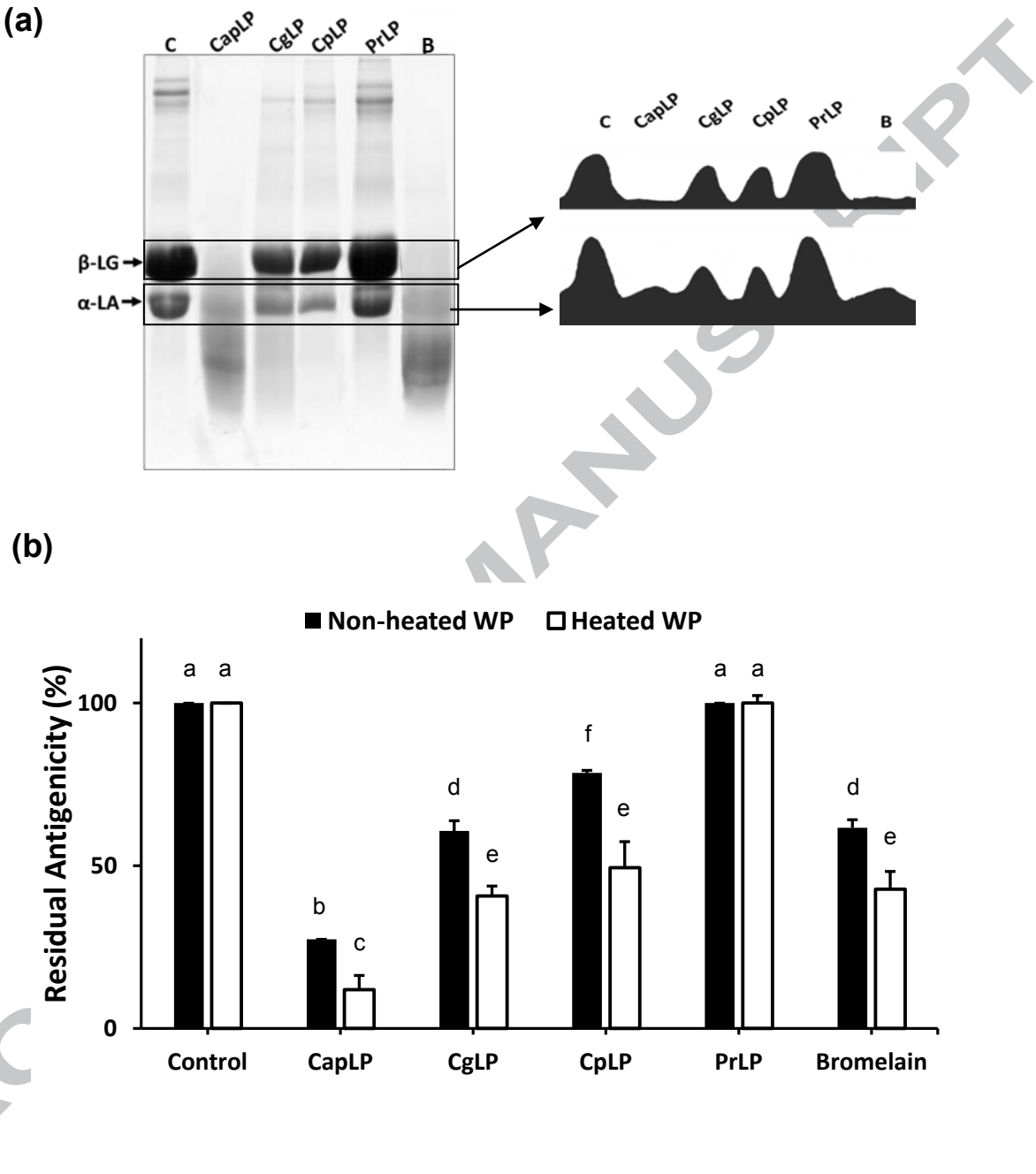

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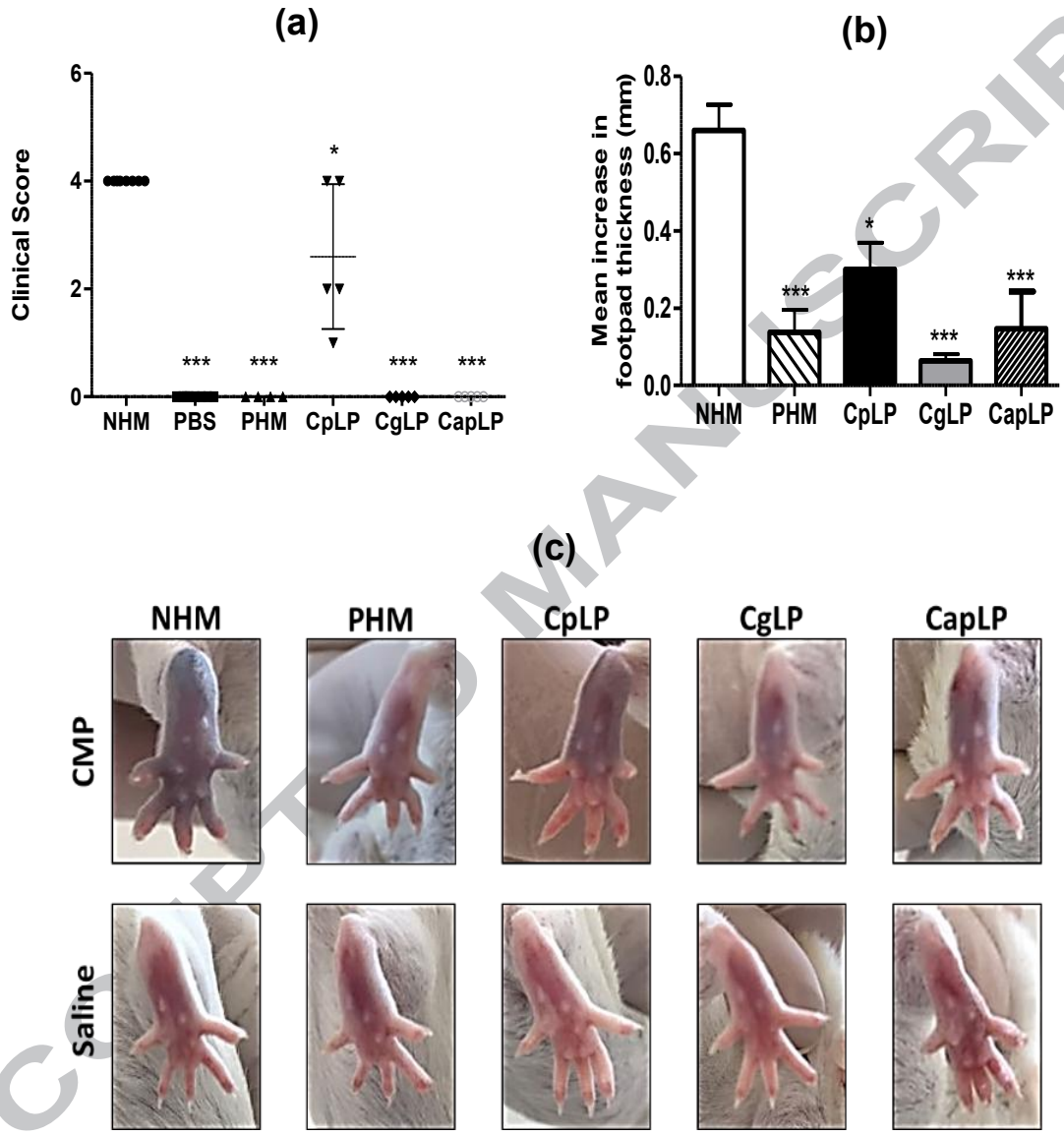

Fig. 5.

Table 1. Clinical scores assigned to trigger the symptoms following the oral challenge.

Score	Symptoms
0	No symptoms
1	Scratching and rubbing around the snout and head
2	Puffiness around the eyes and mouth, piloerection, reduced activity and/or decreased activity with increased respiratory rate
3	Respiratory distress, cyanosis around snout and tail
4	No activity upon stimuli, convulsion
5	Death

Manuscript title: Allergenicity reduction of cow's milk proteins using latex peptidases

João P.B. Oliveira; Angela María Candreva; Gastón Rizzo; Márcio V. Ramos; Jefferson S. Oliveira; Hermógenes D. Oliveira, Maria B. Ary, Guillermo Docena; Cleverson D.T. Freitas

Highlights

- ✓ Latex peptidases were able to perform total hydrolysis of caseins;
- ✓ On the other hand, whey proteins were more resistant to proteolysis;
- ✓ Heat pretreatment of the whey proteins enhanced the degree of hydrolysis;
- ✓ *In vivo* tests showed that latex peptidases reduced antigenicity and allergenicity;
- ✓ Results were similar to a commercial partially hydrolyzed formula