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Gut peptidases from a specialist herbivore of latex plants are capable of milk protein hydrolysis: Inputs for hypoallergenic milk formulas

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ABSTRACT

Transitory allergies to cow milk proteins in infants or adults have become a public health problem. Although extensively or partially hydrolyzed cow milk protein formulas are available, these products are costly. Therefore, studies into innovative enzymes to digest cow milk proteins are needed. *Danaus plexippus* gut peptidases were purified and examined with regard to cow milk protein hydrolysis. The peptidases hydrolyzed caseins and whey proteins. However, after heat treatment, there was a significant improvement in β-lactoglobulin hydrolysis. The hydrolyzed cow milk proteins were not recognized by anti-casein antibodies and only reacted slightly with antibodies against whey proteins. This performance was better than that of partially hydrolyzed formulas and similar to that of an extensively hydrolyzed formula. These results suggest that *D. plexippus* gut peptidases are suitable and innovative enzymes to produce hypoallergenic cow milk protein formulas.

1. Introduction

Milk allergy is an immunological reaction induced by undigested proteins present in milk. It is the most common food allergy during infancy and childhood (Sicherer & Sampson, 2014). Although, in more than 80% of cases the allergy is transitory, this immunological reaction can result in intensified gastrointestinal, respiratory and dermatological symptoms (Fiocchi et al., 2010). Consequently, the supply of energy and amino acids for the growth and development of children is reduced to some extent (Luyt et al., 2014). Although cow milk contains more than 25 different proteins (Hochwallner, Schulmeister, Swoboda, Spitzauer, & Valenta, 2014), caseins, β-lactoglobulin and α-lactalbumin (whey proteins) are reported as the major milk allergens (Monaci, Tregoat, van Hengel, & Anklam, 2006). Therefore, the partial or extensive cleavage of cow milk proteins (CMP) achieved by peptidases is one of the most effective methods to reduce allergic reactions to these proteins (Bu, Luo, Chen, Liu, & Zhu, 2013). Even though very effective, these formulas are expensive, making them unaffordable to many people. Children suffering from milk allergy have few alternative protein sources to supply their daily intake to minimally support their growth and development. Soy protein formulas, which are the immediate substitute for CMP, are not adequate for the nutritional needs of infants and in some cases cross-reactions between antibodies against CMP with soy proteins have been documented (Candreva et al., 2016). To confront this complex scenario, the identification of novel enzymatic sources suitable for efficiently digesting CMP and producing hypoallergenic formulas is needed. Therefore, the study of new peptidases able to hydrolyze milk proteins should be encouraged.

The caterpillar of the Monarch butterfly is extremely well adapted to feed on the leaves of latex-producing plants, such as *Calotropis procera*. A previous study by Pereira and collaborators (2010) showed that *D. plexippus* gut peptidases are able to hydrolyze all proteins present in the latex of *C. procera* within a few minutes. Interestingly, this same protein fraction from *C. procera* latex is toxic to other insects (Ramos et al., 2007). Moreover, *D. plexippus* gut peptidases are resistant to hydrolysis by latex peptidases even after 24 h of incubation (Pereira et al., 2010). Therefore, our hypothesis was that *D. plexippus* gut peptidases can be used as powerful biomolecules to hydrolyze other proteins, including food proteins. Therefore, the aim of the present work was to purify *D. plexippus* gut peptidases by affinity chromatography and evaluate their hydrolytic action on CMP (caseins, β-lactoglobulin and α-lactalbumin) and to compare the resulting hydrolysate with commercial milk formulas.

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2. Materials and methods

2.1. Materials

Acrylamide (17-1302-02), bis-acrylamide (17-1304-02), sodium dodecyl sulfate (SDS) (17-313-01), HiTrap™ Benzamidine Sepharose 4 Fast Flow column (17-5143-01), HiTrap DEAE FF Sepharose Fast Flow (17-5055-01), HiTrap CM FF Sepharose Fast Flow (17-5056-01), HiTrap Protein A Sepharose high performance column (17-0402-01) and low molecular mass markers (17-0446-01) were acquired from GE Healthcare Life Science (São Paulo, SP, Brazil). Sequencing-grade modified trypsin was obtained from Promega (V511B) (São Paulo, SP, Brazil). Azocasein (A2765), porcine pepsin (P7012), Freund's complete (F5881) and incomplete adjuvant (F5506), goat anti-rabbit IgG conjugated with alkaline phosphatase (A6066) and *p*-nitrophenylphosphate disodium (N2765) were obtained from Sigma-Aldrich (São Paulo, SP, Brazil). All other chemicals were of analytical grade.

2.2. Gut protein extract

Gut protein extracts of *Danaus plexippus* larvae (5th instar) were obtained according to Pereira et al. (2010), dialyzed against distilled water, lyophilized and stored at −20 °C until further assays were performed. Soluble proteins were quantified by Bradford's method (Bradford, 1976).

2.3. Gut peptidase purification

D. plexippus gut peptidases (DpGp) were purified by affinity chromatography using a HiTrap™ Benzamidine Sepharose 4 Fast Flow column coupled to an AKTA chromatography system (GE HealthCare). The unbound proteins (PI) were washed with equilibrium buffer (50 mM Tris-HCl pH 7.4, containing 0.5 M NaCl) and the bound proteins (PII, DpGp) were eluted with 50 mM glycine-HCl (pH 3.0) at a flow rate of 1 mL/min. Proteins were monitored at 280 nm. Both peaks (PI and PII) were dialyzed against distilled water and lyophilized.

In order to purify the peptidases, the PII fraction from the HiTrap™ Benzamidine Sepharose 4 Fast Flow column was submitted to ion exchange chromatography in DEAE-Sepharose fast flow and CM-Sepharose fast flow columns, which were previously equilibrated with 25 mM Tris-HCl buffer (pH 7.0). The proteins were eluted from the column by using a linear gradient from 0 up to 1 M NaCl in 25 mM Tris-HCl buffer (pH 7.0). Additionally, the PII fraction was also submitted to reversed-phase high-performance liquid chromatography (RP-HPLC). Samples of 50 µL (1 mg/mL) were loaded in the C2/C18 column (µRPC C2/C18 ST, bed length 100 mm, i.d. 4.6 mm, 3 µm particle size, 12 nm porosity, Amersham Bioscience) coupled to a Jasco CO-2060 Plus HPLC device. The proteins were eluted using a linear gradient from 5% to 80% acetonitrile for 30 min, containing 0.1% trifluoroacetic acid (TFA), at 0.5 mL/min flow rate. Proteins were detected at 280 nm.

2.4. Proteomic analysis

2.4.1. One-dimensional gel electrophoresis

Polyacrylamide gel electrophoresis (15%) in the presence of SDS (1D-SDS-PAGE) was performed according to Laemmli (1970). The samples were dissolved in sample buffer [0.0625 M Tris-HCl buffer (pH 6.8) containing 2% SDS] and the runs were performed at 25 mA (25 °C for 2 h) using 10 × 10 cm gels. Proteins were stained using Coomassie brilliant blue R-350.

2.4.2. Protein identification by mass spectrometry (LC–MS/MS)

The protein bands from the 1D-SDS-PAGE gels were removed, destained, and digested with sequencing-grade modified trypsin according to Hellman, Wernstedt, Góñez, and Heldin (1995). The tryptic peptides were analyzed using a Synapt G1 HDMS mass spectrometer (Waters, Manchester, UK) coupled to a Nano UPLC-ESI system, as described by Freitas et al. (2016). Proteins were identified using the NCBI database and MASCOT search engine (Matrix Science Ltd., London, UK; website: http://www.matrixscience.com).

2.5. Proteolytic activity

Total proteolytic activity was evaluated using azocasein as non-specific substrate (Freitas et al., 2007). Briefly, 50 µL of total gut protein extract or peaks from affinity chromatography (1 mg/mL) were mixed with 1% azocasein (200 µL) and the final volume of the reaction was adjusted to 300 µL with 50 mM Tris-HCl (pH 9.0) or 50 mM sodium phosphate (pH 6.5). The reactions were performed at 37 °C for 1 h, and then stopped with 10% TCA solution. The reaction products were measured at 420 nm.

2.6. Autolysis assays

D. plexippus gut peptidases (DpGp) [1 mg/mL in 50 mM Tris-HCl (pH 9.0) or 50 mM sodium phosphate (pH 6.5)] were incubated at different temperatures (4, 25 and 37 °C) and for different time intervals (1, 2, 4 and 24 h). After incubation, aliquots (50 µL) were used to determine the residual proteolytic activity, as described in Section 2.5. Protein autolysis was verified by 15% polyacrylamide gel electrophoreses in the presence of SDS (1D-SDS-PAGE), as described previously in Section 2.4.1.

2.7. Caseins and whey protein purification

Caseins and whey proteins were purified according to Egito et al. (2007). Briefly, raw bovine milk (obtained from a local market) was centrifuged (2100 × *g* at 32 °C for 30 min) and the supernatant (skimmed milk) was separated and acidified with 0.1 M HCl to pH 4.6. Then, caseins and whey proteins were separated by centrifugation at 1500 × *g* at 20 °C for 20 min. The supernatant (whey proteins) was collected and the precipitate (sodium caseinate) was washed three times with distilled water and centrifuged again. Both fractions were dialyzed against distilled water at 4 °C for two days using membranes with an 8000 Da cut-off, and then lyophilized for further use.

2.8. β-Lactoglobulin purification

β-lactoglobulin was purified as described by Kitabatake and Kinekawa (1998). The whey protein fraction (non-lyophilized) was adjusted to pH 2.0 with 1 M HCl and porcine pepsin was added to a ratio of 1:200, enzyme:substrate (mass:mass). After 1 h at 37 °C, the proteins were precipitated overnight with 75% ammonium sulfate and then centrifuged at 20,800 × *g* for 30 min at 25 °C. The supernatant (β-lactoglobulin) was dialyzed against distilled water at 4 °C for two days using membranes with an 8000 Da cut-off, and then lyophilized. The homogeneity of β-lactoglobulin was checked by 15% 1D-SDS-PAGE as described in Section 2.4.1.

2.9. Hydrolysis of milk proteins

The hydrolytic potential of DpGp on the milk proteins (caseins, whey proteins and purified β-lactoglobulin) was assessed by zymograms and *in vitro* assays. For the zymograms, the gels were supplemented with the four protein substrates at 0.2% (m/m): gelatin (control), caseins, whey proteins and purified β-lactoglobulin. After the electrophoretic runs of DpGp, the gels were washed twice with 2.5% Triton X-100 for 30 min to remove all SDS. Afterward, the gels were incubated in 50 mM Tris-HCl (pH 9.0) or 50 mM sodium phosphate (pH 6.5) for 1 h at 37 °C. Protein hydrolysis was detected as transparent bands in blue gels after staining with 0.1% Coomassie brilliant blue R-350.