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Interaction between wheat alpha-amylase/trypsin bi-functional inhibitor and mammalian digestive enzymes: Kinetic, equilibrium and structural characterization of binding

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ABSTRACT

Alpha-amylase/trypsin bi-functional inhibitors (ATIs) are non-gluten protein components of wheat and other cereals that can hypersensitise the human gastrointestinal tract, eventually causing enteropathies in predisposed individuals. These inhibitory proteins can act both directly by targeting specific pro-inflammatory receptors, and indirectly by impairing the activity of digestive enzymes, the latter event causing the accumulation of undigested peptides with potential immunogenic properties. Herein, according to a concerted approach based on *in vitro* and *in silico* methods we characterized kinetics, equilibrium parameters and modes of binding of the complexes formed between wheat ATI and two representative mammalian digestive enzymes, namely trypsin and alpha-amylase. Interestingly, we demonstrated ATI to target both enzymes with independent binding sites and with moderately high affinity.

1. Introduction

Carbohydrates, proteins, lipids, and minerals contained in cereals represent a fundamental dietary sustenance for world population. Nevertheless, cereal consumption can be associated with allergies and other disorders in predisposed individuals (Gasbarrini & Mangiola, 2014; Marchioni Beery & Birk, 2015). Among these conditions, celiac disease, a T cell-mediated inflammatory intestinal enteropathy caused by dietary gluten or other similar proteins, affects nearly 1% of population (Allen, 2015; El-Salhy, Hatlebakk, Gilja, & Hausken, 2015; Ontiveros, Hardy, & Cabrera-Chavez, 2015; Serena, Camhi, Sturgeon, Yan, & Fasano, 2015). Gluten embraces a group of water-insoluble proteins, which are classified as gliadins and glutenins, and other water-soluble proteins (Shewry, Halford, & Lafiandra, 2003). Structurally, because of their high content in proline and glutamine, gliadins and glutenins share partial-to-extensive resistance to degradation by major human gastrointestinal proteases (due to the lack of adequate cleavage-site specificity (Shan et al., 2002)), resulting in the accumulation of incompletely degraded peptides with potential immunogenic properties. In fact, these oligopeptides can be sensed by the intestinal immune system and trigger adverse responses.

Among non-gluten proteins, alpha-amylase/trypsin bi-functional inhibitors (ATIs) are non-conventional gastrointestinal sensitizing agents (Tatham & Shewry, 2008). Specifically, they are albumin proteins that can increase the levels of gluten-like immunogenic peptides as the result of the inhibition of digestive enzymes and the consequent impaired degradation of dietary cereal proteins. In fact, being plant defense proteins against parasites, ATIs are intended to block the activity of exogenous enzymes from digesting seed carbohydrates and proteins (Gadge et al., 2015). Beside the direct inhibition of digestive enzymes, ATIs can also stimulate the release of pro-inflammatory cytokines in monocytes, macrophages, and dendritic cells (Junker et al., 2012). Collectively, these evidences labeled cereal ATIs as multifaceted contributors to immune activation in celiac disease, and postulated ATIs may stimulate and prolong inflammation and immune reactions in a number of intestinal and non-intestinal immune disorders, among these non celiac gluten sensitivity.

In this study, we kinetically and structurally dissected the molecular basis of the interaction between wheat ATI and two representative mammalian digestive hydrolases, namely trypsin and alpha-amylase, according to a concerted approach based on computational, spectrophotometric and biosensor studies.

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2. Materials and methods

2.1. Materials

Tris(hydroxymethyl)aminomethane (Tris), NaH_2PO_4, Na_2HPO_4, NaCl, HCl, NaOH and CH_3COONa were all obtained from Mallinckrodt Baker (Milan, Italy). 2-(N-Morpholino)ethanesulfonic acid (MES), dimethylsulfoxide (DMSO), $MnCl_2$, $CaCl_2$, $CuSO_4$, imidazole, KCl, Tween-20, potassium thiocyanate (KSCN), porcine trypsin, trypsin substrate N-alpha-benzoyl-L-arginine-*p*-nitroanilide (L-BAPNA), sucrose, porcine alpha-amylase and amylase substrate 2-chloro-4-nitrophenyl-a-maltotrioside were all obtained from Sigma-Aldrich (Milan, Italy). N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), ethanolamine and carboxylate cuvettes were obtained from Farfield Group (Cheshire, UK). All reagents and chemicals were of the highest purity available. Seeds from wheat (*Triticum turgidum L.* subsp. *durum*, Iride variety) were obtained from a local store and from Cermis (Centro Ricerche e Sperimentazione per il Miglioramento Vegetale "N.Strampelli" – Tolentino (MC)). The Cary 1E UV–vis spectrophotometer was obtained from Varian (Palo Alto, CA). The IAsys Plus Biosensor came from Thermo Fisher Scientific (Milan, Italy). The FPLC System AKTA equipped with a UV–vis detector, and His-trap Metal Chelating Columns were obtained from GE Healthcare (Milan, Italy). Gel filtration analyses were performed on a Tosoh Progel™-TSK G2000 SWXL column, 30 cm × 7.8 mm (Sigma Aldrich, Milan, Italy).

2.2. Purification of ATI

Wheat seeds were crushed into fine powder using a blender/tissue crusher. Powdered samples were extracted in 50% isopropanol (1:5 weight-to-volume ratio) for 30 min under gentle stirring at room temperature as previously reported (van den Broeck et al., 2009). Extraction conditions were optimized on the basis of different experiments on the variations of the composition of the extracting solution, temperature and the duration of the extraction time. After this step, the suspension was centrifuged at 2500 rpm for 15 min at room temperature to remove debris. The supernatant containing the species of interest was collected and concentrated 10-fold by centrifugal evaporation under vacuum using a Centrivap device (Labconco), equipped with a cold trap.

Finally, ATI was purified by IMAC on a ÄKTA Basic chromatographic system using HiTrap Chelating HP columns charged with Cu(II) ions according to an adaptation of the method described by Roy and Gupta (2000). The sample was applied at 1 column volume/min flow rate, and then the column was washed with 5 column volumes (CV) of binding buffer (0.02 M sodium phosphate, 0.5 M NaCl, 40 mM imidazole, pH 7.4) to remove non-tightly or non-specifically bound species. ATI was eluted using a linear gradient (0–100%, 7 CV) of elution buffer (0.02 M sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4). Fractions with both trypsin and amylase inhibitory activities were dialyzed (molecular mass exclusion 3 kDa – Spectra/Por) to remove imidazole and lower molecular weight species, freeze-dried by under-vacuum sublimation, and finally stored at −20 °C till use. Protein content was estimated using Coomassie blue-based reagent (Bradford, 1976). ATI molecular weight was determined by gel filtration chromatography: briefly, ATI-containing fraction was injected into the AKTA HPLC system equipped with a Tosoh Progel™-TSK G2000 SWXL column, and eluted with an isocratic mobile phase consisting of 0.1 M $NaSO_4$, 0.1 M NaH_2PO_4, pH 7 (flow rate: 1 mL/min, wavelength: 280 nm). The purity (>95%) was assessed according to Papadoyannis and Gika (2005).

2.3. Binding assay

Kinetic and equilibrium parameters of interaction between ATI and two representative mammalian hydrolases were determined according to a biosensor-binding assay as previously described (Cuccioloni et al., 2011). Briefly, carboxylate-functionalized cuvettes were sequentially washed with PBS-T (10 mM Na_2HPO_4, 2.7 mM KCl, 138 mM NaCl, 0.05%(v/v) Tween-20, pH 7.4), and detergent-free PBS, pH 7.4. Finally, the carboxylic surface was activated with an equimolar solution of EDC and NHS (Edwards, Lowe, & Leatherbarrow, 1997).

Both trypsin and amylase were dissolved in 10 mM CH_3COONa buffer, pH 5, to a final concentration of 1 mg/mL, and independently incubated onto dedicated carboxylate surfaces for at least 15 min. Non-efficiently-bound enzyme was washed out with PBS, and non-reacted carboxylic groups were deactivated with 1 M ethanolamine, pH 8.5, prior to any addition of soluble ligands. ATI was added at increasing concentrations in the range

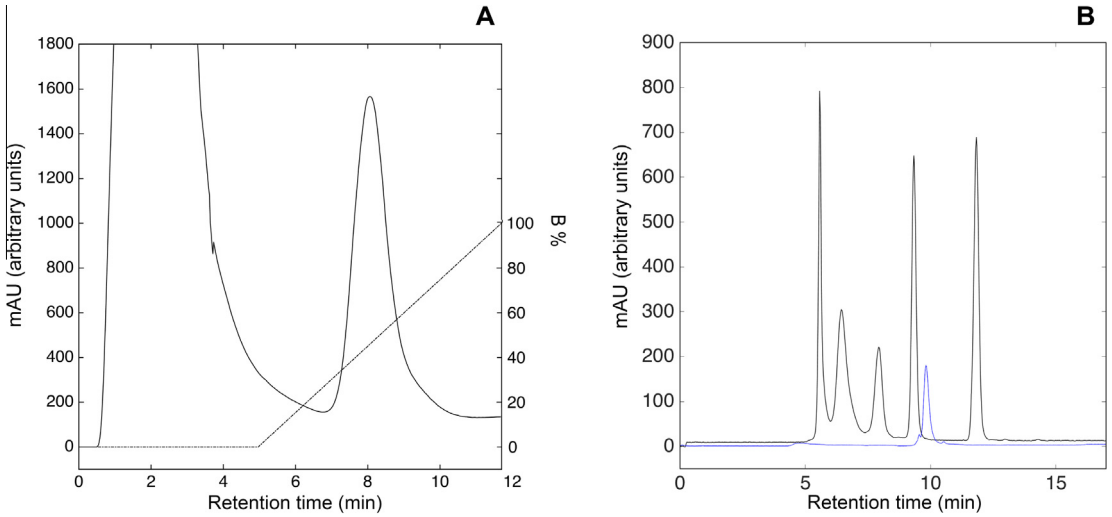

Fig. 1. Elution chromatogram of the 50% isopropanol extracts (Panel A): the peak at nearly 8 min corresponds to ATI-containing fraction; dotted line indicates elution gradient (see Section 2). Gel filtration profile of the peak containing ATI after dialysis (blue curve) superimposed to molecular weight markers (Thyroglobulin, MW = 670 kDa; γ-globulin, MW = 158 kDa; ovalbumin, MW = 44 kDa; Myoglobin, MW = 17 kDa; vitamin B12, MW = 1.35) (Panel B).