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A novel catalysis by porcine pepsin in debranching guar galactomannan

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ABSTRACT

Background: Pepsin (porcine stomach mucosa, E.C. 3.4.23.1), an acid protease catalyzes the hydrolysis (debranching) of guar galactomannan (GG), a co-polymer of mannose and galactose residues thereby showing its non-specific catalysis towards glycosidic substrates.
Results and conclusions: Use of non-specific inhibitors, chemical modification agents and peptide mapping of native and GG – bound pepsin upon proteolytic digestion with *Staphylococcus aureus* V8 protease revealed the involvement of Asp^{138} residue in the catalysis, which was confirmed by computational modelling studies.
General significance: Here we show a novel mode of catalysis (other than proteolysis) by porcine pepsin with a different active site residue.

1. Introduction

GG, a branched co-polymer of mannose and galactose residues, obtained from the seeds of leguminous plant *Cyamopsis tetragonalobus* finds numerous applications in various food and non-food industries, and therefore is of high commercial importance (Greenberg & Slavin, 2003; Kokol, 2002). High branching, high viscosity and high molecular weight of GG, nevertheless, restricts additional uses of GG, which was partly overcome by selective removal of side chain galactose residues using α-galactosidase. Though successful, the utility of this technology is restricted because of the laborious steps involved and cost effectiveness for isolating the pure enzyme. A few earlier reports claiming the multiple specificity of several enzymes on various carbohydrate macromolecules (Vishu kumar, Gowda, & Tharanathan, 2004; Pantaleon, Yalpani, & Scollar, 1992; Shobha, Vishu Kumar, Tharanathan, Rathna, & Gaonkar, 2005), emerged as an alternative approach for the application of non-specific enzymes in modifying guar galactomannan. Fu, Wu, Chang, and Sung (2003) also isolated and characterized three chitosanase isozymes from porcine pepsin, and showed that 14 out of first 15 N-terminal amino acid residues of isozyme PSC-III were identical with those of pepsin B.

In this study, an attempt has been made to unravel why and how pepsin – a proteolytic enzyme brings about catalysis of structurally unrelated carbohydrate substrates. The use of site specific inhibitors, chemical modification agents and peptide mapping of native and GG-bound pepsin on proteolytic digestion with Glu-C V8 protease were carried out to know the binding site residue and also to elucidate the mode of action of pepsin in debranching of GG. Pepsin, a monomeric β-protein (M_r, 34.6 kDa) with two domains comprising a high percentage of acidic residues (43 out of 327 are aspartic and glutamic acids, pK_a – 1.5), of which Asp^{32} (ionized) and Asp^{215} (unionized) are mainly responsible for proteolysis. Though, functionally a proteolytic enzyme, the non-specific reactivity of pepsin towards glycosidic substrates may be due to structural features mimicking several carbohydrate degrading enzymes, which are characterized by the presence of two domains with barrel-like structures having one end of the barrel wider than the other. At the wider end is an elongated cleft, which would become narrower or wider depending on the size of the substrate (Jedrzejas, 2000). The pairwise sequence alignment of porcine pepsin (5 pep) with guar α-galactosidase showed 25% sequence homology (Fig. 1), whose

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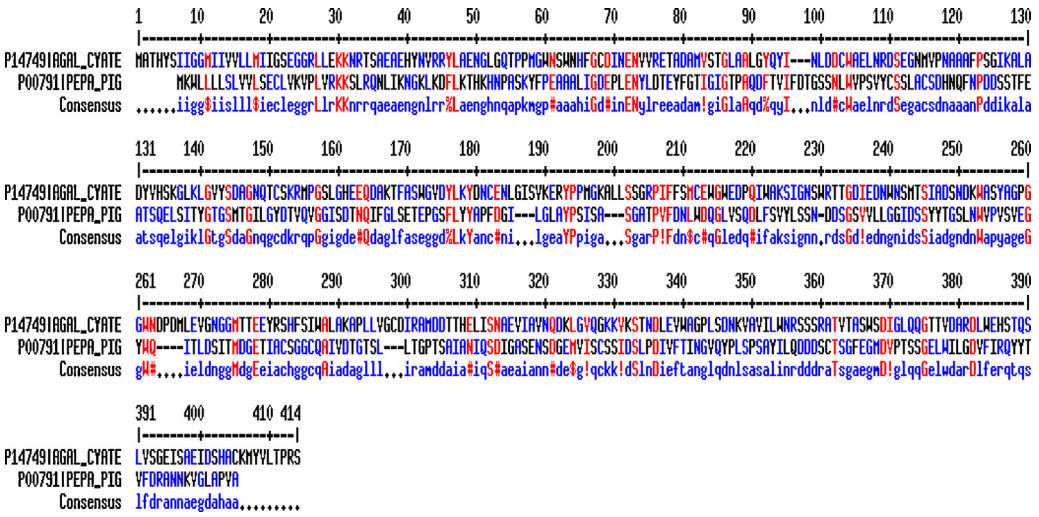

Fig. 1. Multiple alignment of porcine pepsin and guar α-galactosidase.

active sites contain an Asp residue involved in the catalysis of their cognate substrates.

2. Materials and methods

2.1. Materials

Pepsin (porcine stomach mucosa, E.C. 3.4.23.1), haemoglobin (Bovine), pepstatin, phenylmethylsulfonylketone (PMSF), N-tosyl-L-lysine-chloromethylketone hydrochloride (TLCK), iodoacetate, N-(3-methyl amino propyl) N′-ethylcarbodiimide hydrochloride (EDAC), glycine methyl ester (GME), endoproteinase Glu-C (*Staphylococcus aureus* strain V8, E.C. 3.4.21.19) and Sephadex G-100 were from Sigma Chemicals Co., St. Louis, MO, USA. Guar gum was a gift from Hindustan Gum Chemicals, Haryana, India. All other reagents and chemicals were of highest purity available.

2.2. Gel permeation chromatography (GPC)

The crude enzyme solution was loaded onto Sephadex G-100 column (100 cm length *x* 0.7 cm i.d.) and eluted with 50 mM acetate buffer of pH 5.6. Fractions, analyzed at 280 nm, which showed maximum proteolytic activity and also activity towards GG were pooled.

2.3. Native/SDS PAGE

Native and SDS-PAGE was done on 7.5% gel according to the method of Laemmli (1970) and the isolated protein bands were visualized by staining with Coomassie Brilliant Blue (CBB).

2.4. Zymogram analysis

2.4.1. Proteolytic activity

In addition to the various analytical methods reported elsewhere, the zymogram analysis of pepsin towards specific substrate was carried out according to Lopez, Lopez, Lopez, Carreno, and Toro (1998). After electrophoresis (acid PAGE), the gel was immersed in 0.1 M HCl to reduce the pH to 2.0. After 15 min, the gel was immersed in a solution containing 0.25% haemoglobin in 0.1 M glycine–HCl, pH 2.0 at 4 °C for 30 min, then for 90 min in a fresh haemoglobin solution at 37 °C. The gel was washed with distilled water and fixed for 15 min in 12% trichloroacetic acid (TCA), and then stained with 0.1% Coomassie brilliant blue in methanol–glacial acetic acid–water (5:2:5). Destaining was carried out using an aqueous solution of 30% methanol–10% acetic acid.

2.4.2. Activity towards GG

After electrophoresis the gel was incubated with 0.5% GG at 40 °C for 8 h in acetate buffer (0.1 M) of pH 5.5 and later stained with 0.1% Congo red (1 h), excess stain was removed using 1 M potassium chloride.

2.5. MALDI-TOF-MS

The purified pepsin was mixed with an equal volume of matrix (α-cyano-4-hydroxycinnamic acid prepared in CH_3CN:H_2O:TFA, 80:20:0.1), dried at 25 °C under atmospheric pressure and transferred into the vacuum chamber of the mass spectrometer (Compact Analytical SEQ MALDI-TOF-MS, Kratos, UK) in a reflective positive ion mode.

2.6. Automated gas-phase protein sequencing

The N-terminal sequencing of purified pepsin was carried out on Applied Biosystems 491A automated gas phase protein sequencer (Procise 491A) by Edman degradation using the standard protocols.

2.7. Enzyme assay

Proteolytic activity of purified pepsin was assayed spectrophotometrically using a Shimadzu UV-Visible spectrophotometer at 280 nm, by incubating with haemoglobin (2.5%) at pH 1.5–2.0, for 1 h, 37 °C, and estimating the TCA-soluble peptides released. Specific activity was expressed as one unit = absorbance at 280 nm/reaction time × mg protein in the reaction mixture.

Non-specific hydrolytic activity was assayed using GG (0.5%, w/v) solution containing pure pepsin (100 μg), incubated for 1 h at 37 °C (Shobha & Tharanathan, 2008). The reducing sugar, released into the supernatant was estimated by the ferricyanide method (Imoto & Yagishita, 1971). One unit of activity was expressed as μmoles of reducing equivalents released min^{-1} mg^{-1} protein.

2.8. Galactose to mannose ratio (G/M)

The G/M ratio of pepsin treated GG was evaluated, after acid hydrolysis followed by derivatization into alditol acetate, by GC on OV-225 (3% on Chromosorb W) column connected to Shimadzu 8A