Carbohydrate Polymers 102 (2014) 615–621

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

A novel catalysis by porcine pepsin in debranching guar galactomannan

Mysore S. Shobha [a], Lalitha R. Gowda [b], Rudrapatam N. Tharanathan [a,*]

[a] Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Council of Scientific and Industrial Research, Mysore 570 020, India

[b] Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Council of Scientific and Industrial Research, Mysore 570 020, India

ARTICLE INFO

Article history:
Received 9 April 2013
Received in revised form
16 November 2013
Accepted 27 November 2013
Available online 4 December 2013

Keywords:
Porcine pepsin
Guar galactomannan
Non-specificity
Peptide mapping
Active site
Docking

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

* Corresponding author. Tel.: +91 080 23198195.
E-mail address: tharanathan@yahoo.co.uk (R.N. Tharanathan).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.11.043

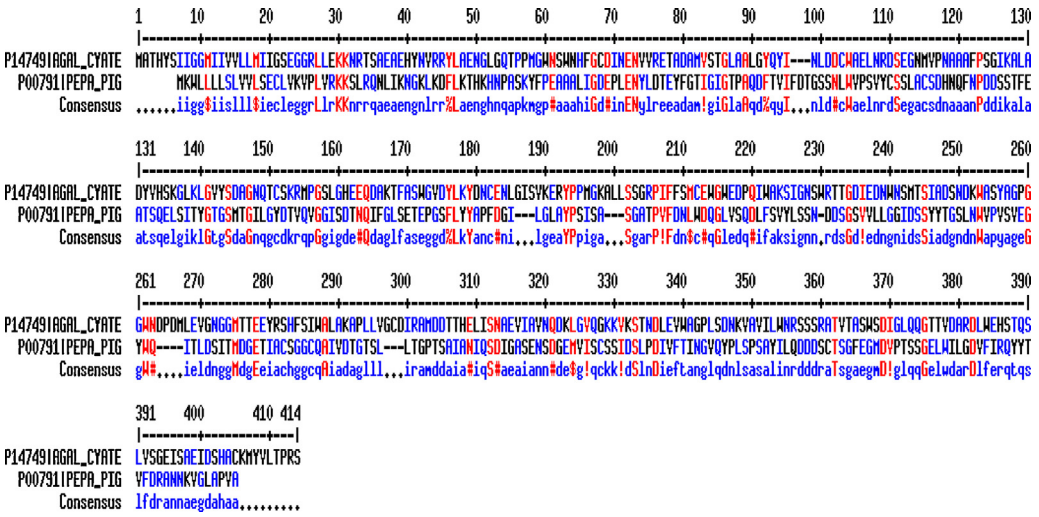

Fig. 1. Multiple alignment of porcine pepsin and guar α-galactosidase.

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

equipped with flame ionization detector (Sawardekar, Slonekar, & Jeanes, 1965).

The hydrolysis of GG was further studied in the concentrated supernatant of enzyme digest by HPLC on a precalibrated (using galactose and mannose, 10 μL) μ-Bonda pack aminopropyl column (4.1 mm × 300 mm) using acetonitrile:water (75:25) mixture as mobile phase at a flow rate of 1 mL min^{-1} and RI Detector.

2.9. Fluorescence studies

Fluorescence measurements were recorded on a Shimadzu RF 5000 spectrofluorimeter using 5 nm path length at 27 °C. Fluoresence emission spectra were recorded (300–450 nm) with the excitation wavelength set at 280 nm. Appropriate blanks were used for baseline correction of fluorescence intensity. For fluorimetric titration, 3.0 mL of solution with appropriate concentrations of pepsin (100 μg mL^{-1}) were titrated in acetate buffer (0.1 M) of pH 5.5 by successive addition of GG solution (0–0.5%).

2.10. Inhibitory studies

The inhibitory effects of pepstatin, PMSF, TLCK and iodoacetate were carried out by pre-incubating purified pepsin with the inhibitors at varying concentration (2–10 mM) for 1 h at 45 °C. The residual protease activity and activity towards GG were assayed.

2.11. Chemical modification of carboxyl residues

Carboxyl residue modifications were performed at 25 °C with pepsin in 0.05 M sodium acetate (pH 4.8), containing 0.275 M GME and 0–0.3 M EDAC (Hoare & Koshland, 1967). EDAC and GME were dissolved in water immediately before use and inactivation initiated by the addition of EDAC. A control experiment of enzymes and the nucleophile GME in buffer was run simultaneously, which corresponds to 100% pepsin activity towards GG. Aliquots at 10 min intervals were removed for residual activity determination. The pseudo first order rate constants for the inactivation were determined by a plot of log% residual activity against time. The inactivation kinetics was fitted to the equation: log (% residual activity) = $-k_i\ t$, where k_i is the pseudo first-order inactivation rate constant for a given concentration of EDAC and t is time of inactivation. The inactivation order (n) was calculated from the equation: $\log K_i = n \log[\text{inactivator}] + K_i$, where $\log K_i$ is the second-order inactivation constant. The stoichiometry of the inactivation reaction was determined by a plot of $\log K_i$ versus log(EDAC). The slope of the curve represents the stoichiometry.

2.12. Peptide mapping of GG binding site of pepsin

Native and GG-bound pepsin were incubated with endoproteinase Glu-C from *S. aureus* strain V8 (100:3, w/w) in 0.1 M phosphate buffer, pH 7.8 for 18 h at 37 °C. The proteolytic digestion was arrested by heating the reaction mixture for 20 min in a boiling water bath. The digest was concentrated to dryness and redissolved in 0.5 mL of 0.1% TFA (Matsudaira, 1989). The peptides released were then separated by Rp-HPLC using a C-18 column (4.6 mm × 150 mm; 5 μm) on a Shimadzu LC-10 system equipped with a binary pump using Solvent A (0.1% TFA in water) and solvent B (70% CH_3CN containing 0.05% TFA) (Mahoney & Hermodson, 1980). at a flow rate of 0.7 mL min^{-1} with a linear increase in the solvent strength from 0 to 100% in 85 min (Hermodson & Mahoney, 1983). The peptides were detected at 230 nm. The peptide profiles were compared and analyzed with those derived from native pepsin. A distinct peptide was collected and its sequence determined by Edman degradation. The amino acid sequence of porcine pepsin was retrieved from Swiss-Port/TrEMBL (Acc.No. P00791) and its sequence alignment with that of α-galactosidase was carried out using CLUSTAL X (Higgins, Thompson, & Gidson, 1996) with subsequent gap adjustments. The model for porcine pepsin was derived from the co-ordinates of the structure labelled 5pep in the RCSB Protein Data Bank, which represents pepsin at 2.3 Å resolution (Cooper, Khan, Taylor, Tickle, & Blundell, 1990). The ligand GG (with 6 residues) was constructed and submitted to the PRODRG site (Schuettelkopf & van Aalten, 2004). The initial geometry and topologies were retrieved. The ligand was docked against porcine using Arguslab (Thompson, Planaria software) with a grid spacing of 0.4 Å. The best docked calculations and graphical manipulations were performed using pyMol.

3. Results and discussion

3.1. Criteria of homogeneity

As the commercial enzyme preparations usually are admixed with inorganic substances, used as enzyme stabilizers, attempts were made to purify them before undertaking further detailed structural studies, in order to rule out the involvement of these contaminants in non-specificity.

The commercial preparation of porcine pepsin on native PAGE (Fig. 2A) showed a major protein band along with the presence of a few minor bands. Thus in order to rule out the possible contribution of these minor bands for their non-specific action towards GG the enzyme preparation was purified by preliminary size exclusion chromatography. The protein fraction that exhibited maximum catalytic activity towards both protein and GG was pooled, which upon native and SDS-PAGE showed a single protein of $M_r \sim 34{,}900 \pm 1200$ Da (determined by standard molecular weight markers) corresponding to the earlier major protein band of the crude pepsin, thus ruling out the involvement of contaminants in catalytic action (Fig. 2A). Capillary zone electrophoresis (CZE) and Rp-HPLC profiles also established the existence of a single protein. The N-terminal sequence of the purified protein by Edman degradation revealed Ile-Gly-Asp-Glu-Pro-Leu-Glu-Asn-Tyr-Leu-Asp-Thr-Glu-Tyr-Phe which was identical to the sequence of porcine pepsin reported earlier (Sepulveda, 1973). MALDI-TOF-MS also revealed a single peak of M_r 34,511 Da (Fig. 2B), which was in concordance with the reported molecular weight of porcine pepsin. These results, taken together established the homogeneity of the enzyme beyond ambiguity. The zymogram analysis of the purified pepsin on acid-PAGE for proteolytic activity and activity towards GG showed a single clearing zone confirming the dual role of pepsin in both proteolysis and debranching of GG (Fig. 3).

The effect of varying pepsin concentration was directly proportional to the increased rate of catalysis with first order reaction kinetics upto a concentration of 100 μg, following zero order, and hence for further study, an enzyme:substrate ratio of 1:100 was employed. The enzyme was stable up to 5 h by retaining 90–95% activity, showed maximum stability at pH 5.5 and 45 °C with K_m and V_{max} values of 5.0 mg mL^{-1} and 2790 nmoles min^{-1} mg^{-1}, respectively (Table 1). Various authors (Roncal, Oviedo, Lopez de Armentia, Fernandez, & Villaran, 2007; Vishu Kumar, Varadaraj, & Tharanathan, 2007) also report the hydrolysis of chitosan by pepsin at pH 4.5 and 5.0 respectively. Schlamowitz and Peterson (1959) suggest the pH dependent broad specificity for the action of pepsin on proteins at mildly acidic range (i.e. pH 4 to higher). Campos and Sancho (2003) states pepsin (pH 4–6.5) still retaining its conformation and substrate binding site similar to its original conformation at lower pH. Further reports from Monu, Prasanna Kumari, Vikash Kumar, Pinky, and Jagannadham (2009) and Kamatari, Dobson, and Konno (2003) also substantiate the native conformation of pepsin at pH 5.6 by NMR and CD measurements. Thus, though the enzyme at

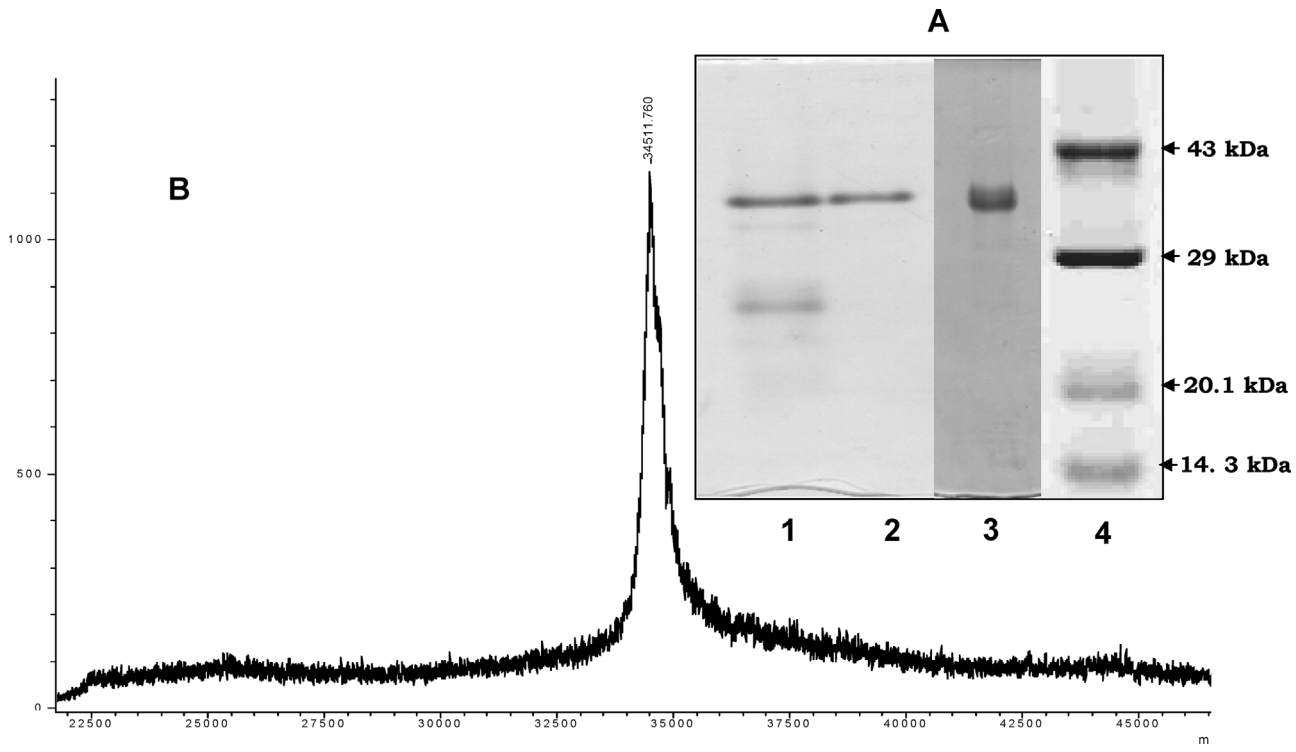

Fig. 2. (A) Native PAGE of crude pepsin (Lane 1) and purified pepsin (Lane 2); SDS PAGE of purified pepsin (Lane 3) and protein markers (Lane 4) (B) MALDI-TOF-MS of purified pepsin.

this pH range shows changes in the proteolytic activity, the overall insignificant changes in the structure and other biophysical properties might favour the enzyme binding to another substrate like carbohydrate, which has made this enzyme to have broad substrate specificity. The pH dependent broad specificity of pepsin at mildly acidic pH probably may lead to the expansion of protein allowing the compact active site to stretch a bit due to non-specific long range interaction between different protonated groups (Goto & Fink, 1989). Such a changes at acidic pH from a largely unfolded state to an intermediate conformational state have been reported for cytochrome C (Ohgushi & Wada, 1983) and human interferon γ (Arakawa, Hsu, & Yphantis, 1987). Fluorescence titration of GG with pepsin resulted in an increased quenching as the concentration of the former increased, suggesting the hydrolytic action of the pepsin.

The GLC analysis of the pepsin treated GG revealed a G:M ratio of 1:3.6 almost similar to LBG (data not shown). Thus the change in the G:M ratio agreed well with the action of pepsin towards GG. Also the HPLC characterization of the supernatant showing the presence of galactose alone further supported the debranching action of pepsin on GG.

3.2. Identification of GG binding site of pepsin

Pepstatin (3 mM), an inhibitor specific to aspartate proteases, totally inhibited the proteolytic activity without altering the activity towards GG. Thereby suggesting different active site residues involved in hydrolysis of GG by pepsin. The addition of PMSF and

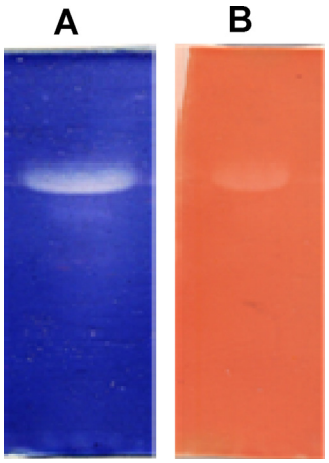

Fig. 3. Zymogram analysis of pepsin on (A) acid PAGE for proteolytic and (B) GG.

TLCK had no effect on both the activities, thereby ruling out the contribution of serine, cysteine and histidine residues in the catalysis of GG. All these results are suggestive of a different carboxyl group, other than that required for proteolysis, in the catalysis of GG. It is well documented that polysaccharide degrading enzymes exhibit acid–base catalytic mechanism, comprising a pair of carboxyl residues, of which one is protonated and the other unprotonated.

Table 1
Kinetic parameters of pepsin towards GG.

Enzyme	pH optimum	Temperature optimum (°C)	K_m (mg mL^{-1})	V_{max} (nmoles min^{-1} mg^{-1} protein)
Pepsin	5.5	45	5.0 ± 0.088	2790 ± 32.8

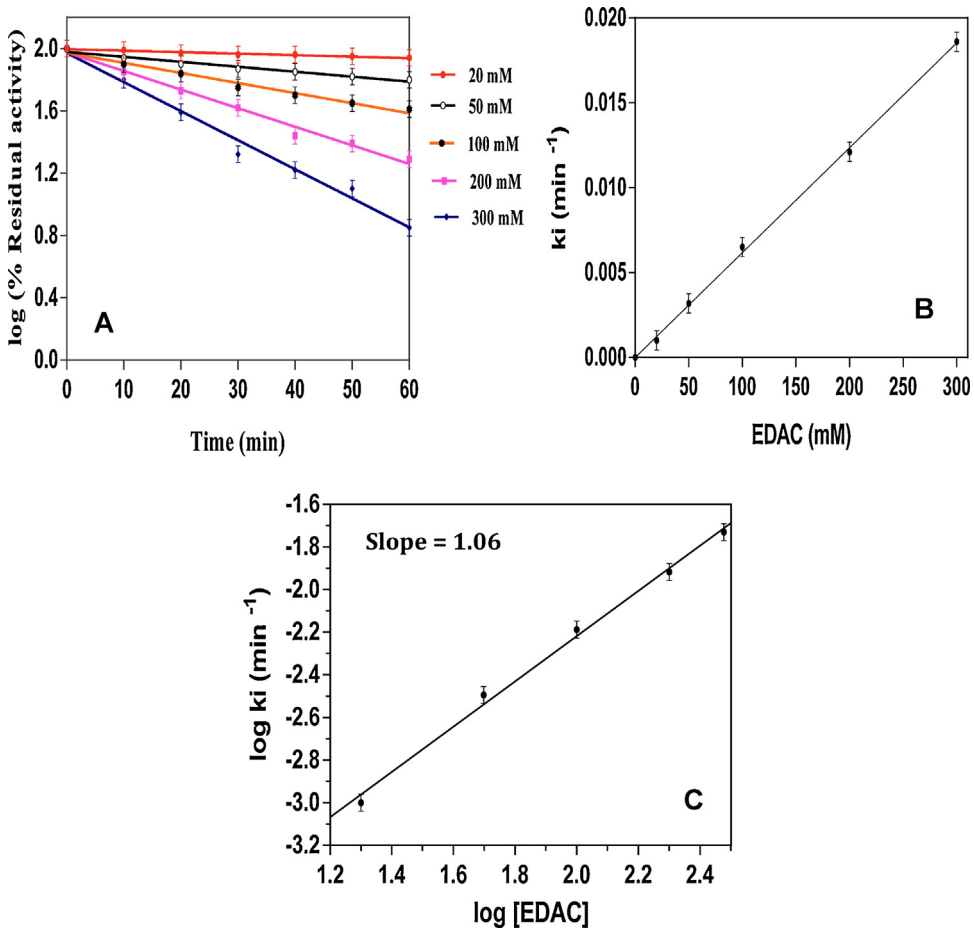

Fig. 4. Inactivation of pepsin by EDAC and GME. (A) Plot of log (% residual activity) versus time. (B) Plot of pseudo first order inactivation rate constant as a function of EDAC concentration. (C) Double logarithimic plot of pseudo first order inactivation rate constant as a function of EDAC concentration.

Incubation of pepsin with either GME or EDAC, specific for modification of carboxylic groups resulted in no inhibition of the enzyme activity (Fig. 4). However the requirement of a relatively high concentration of EDAC for the inactivation is probably due to the high content of carboxylic groups in pepsin. A similar requirement has been reported for various enzymes (Kanade, Paul, Appu rao, & Gowda, 2006; Nyvall, Pedersen, Kenne, & Gacesa, 2000). The semi-logarithmic plot of residual activity at various EDAC concentrations versus time was linear indicating that the inactivation followed pseudo first-order kinetics. A plot of the first-order inactivation rate constant (k_i) against EDAC concentration was also linear. A plot of $\log k_i$ versus log[EDAC] yielded a slope of 1.0613. Since the stoichiometry was near to 1.0 with respect to EDAC, it suggested the involvement of a single carboxylate group in the catalytic activity of pepsin towards GG.

In order to identify the GG binding site, the purified pepsin, subjected to proteolysis with Glu-C V8 protease in the presence and absence of GG, was analyzed by Rp-HPLC (Fig. 5A and B). A distinct peak with a retention time of 27.5 min was detected in the peptide profile of pepsin digested with the enzyme in the presence of GG. The sequence of this peptide, as determined by Edman degradation was NH_2-NLWDQGLVSQ corresponding to the residues 139–148 of porcine pepsin. In other words, the binding of GG with pepsin protected the Asp^{142} residue from proteolysis, which substantiated the loss of enzyme activity upon chemical modification with EDAC.

3.3. Docking of GG with pepsin

Docking simulations use computational methodologies to mimic the biochemical process of a ligand binding in protein-ligand interaction studies. Fig. 6 shows computational simulation model of GG interacting with pepsin, which revealed Asp^{138} to be involved in catalyzing the removal of galactose residues. The GG–pepsin complex showed a mean binding energy of -6.5 kcal mol^{-1} with Asp^{138} residue at a distance of 6.4 Å from the glycosidic oxygen of galactose residue. Also, the imidazole of Pro^{126} and the phenyl ring of Phe^{141} of pepsin showed a perfect stacking with galactose and mannose of GG, thereby facilitating effective binding. Liu, Lai, Chou, Chang, and Lyu (2007) report the hydrophobic interaction of aromatic side chain of Trp and Tyr with the glucose ring of β-cyclodextrin influencing the surrounding residues in the binding of ligand. The contribution of Asp^{142} (identified from GG-binding peptide sequence) in debranching of GG is also evident from its close proximity to the substrate in the ligand bound complex. Generally acid catalysis by hydrolases follows a double displacement mechanism which requires two acidic residues in the vicinity of the active site leading to hydrolysis of the substrate. Nevertheless, an alternative mechanism with a single carboxylate residue has also been reported. Weaver, Grutter, and Matthews (1995) discount the need of a second carboxylate group (Asp^{52}) for catalysis by goose egg white lysozyme thereby ruling out the double displacement mechanism. Perrakis (1994) and Terwisscha van Scheltinga, Lalk,

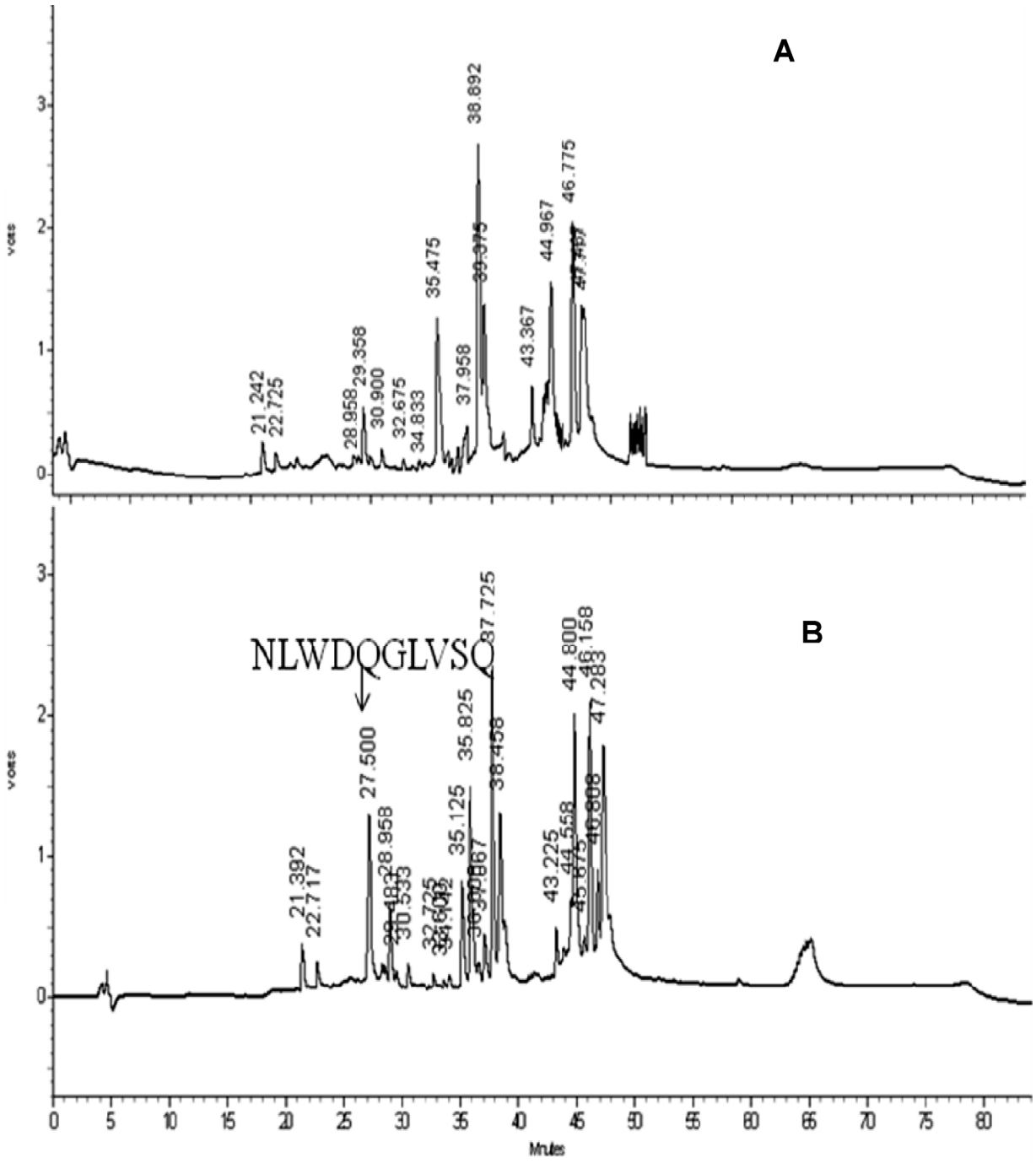

Fig. 5. Rp-HPLC profile of the proteolytic digests of pepsin in the absence (A) and presence (B) of GG.

Beintema, and Dijkstra (1994) report the X-ray crystal structure of family 18 chitinases with no second acidic residue in the active site required to stabilize the oxocarbonium ion.

Based on the above results, a probable mechanism to explain the hydrolytic activity of pepsin towards GG has been proposed. Amist a nonpolar milieu contributed by Val[136], Phe[137] and Leu[140], the protonated Asp[138] donates a proton to the glycosidic oxygen between the C-1 of galactose and C-6 of mannose residue (α-1,6 linkage), which results in the cleavage of the glycosidic bond thereby creating a positive charge on the C-1 of galactose ring resulting in a transient oxocarbonium ion, that is stabilized by the neighbouring negatively charged Asp[142], which is in a polar environment. The oxocarbonium ion intermediate reacts with incoming OH^- of water thereby pushing forward the reaction and also reprotonating Asp[138]. Bjelic and Aqvist (2004) report a similar mechanism involving the direct participation of single catalytic aspartate performing both acid–base functions, with the neighbouring histidine residue providing necessary stabilization along the reaction. The docking simulations of GG with pepsin showed a distance of 6.4 Å between the Asp[138] and glycosidic oxygen and 10.4 Å between Asp[142] and oxocarbonium ion, which is greater than the normally required 3–4 Å. This increased distance may possibly explain the lower activity towards GG when compared to its proteolytic activity. It is also evident that such an increased distance contributes to the slowing down the rate of hydrolysis (Brameld & Goddard, 1998).

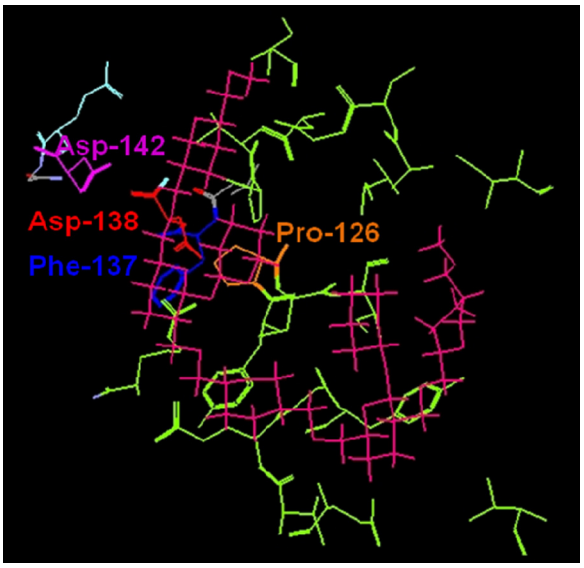

Fig. 6. Details of the interaction between GG and pepsin after automated docking.

4. Conclusion

The present investigation shows that a single protein is involved in the hydrolysis of both specific and other substrates. In view of this, it sounds desirable to know more about the intriguing (non-) specificity of some of the common physiological enzymes. A single enzyme, in addition to hydrolyzing its cognate substrates, depolymerising a variety of unrelated substrates is of biophysiological significance. Such an understanding helps to appreciate better the multifaceted catalytic potential of enzymes in general. Chitosan, an aminopolysaccharide, upon catalysis by pepsin gave oligomeric products of biophysiological importance (Vishu Kumar, Varadaraj, Gowda, & Tharanathan, 2007). The latter holds potential to be used as biological response modifiers for vital applications in medicine.

Acknowledgements

MSS thanks the Council of Scientific and Industrial Research, New Delhi for the Senior Research Fellowship. We acknowledge and thank Proteomic facility, IISc, Bangalore, for the MALDI-TOF-MS spectra.

References

Arakawa, T., Hsu, Y. R., & Yphantis, D. A. (1987). Acid unfolding and self-association of recombinant *Escherichia coli* derived human interferon gamma. *Biochemistry*, *26*, 5428–5432.

Bjelic, S., & Aqvist, J. (2004). Computational prediction of structure, substrate binding mode, mechanism, and rate for a malaria protease with a novel type of active site. *Biochemistry*, *43*, 14521–14528.

Brameld, K. A., & Goddard, W. A., III. (1998). Substrate distortion to a boat conformation at subsite-1 is critical in the mechanism of family 18 chitinases. *Journal of American Chemical Society*, *120*, 3571–3580.

Campos, L. A., & Sancho, J. (2003). The active site of pepsin is formed in the intermediate conformation dominant at mildly acidic pH. *FEBS Letters*, *538*, 89–95.

Cooper, J. B., Khan, G., Taylor, G., Tickle, I. J., & Blundell, T. L. (1990). X-ray analyses of aspartic proteases. II. Three dimensional structure of the hexagonal crystal form of porcine pepsin at 2.3 Å resolution. *Journal of Molecular Biology*, *214*, 199–222.

Fu, J. Y., Wu, S. M., Chang, C. T., & Sung, H. Y. (2003). Characterization of three chitosanase isozymes isolated from a commercial crude porcine pepsin preparation. *Journal of Agricultural and Food Chemistry*, *51*, 1042–1048.

Goto, Y., & Fink, A. L. (1989). Conformational states of β-lactomase: Molten-globule states at acidic and alkaline pH with high salt. *Biochemistry*, *28*, 945–952.

Greenberg, N. A., & Slavin, J. L. (2003). Partially hydrolysed guar gum: Clinical nutritional uses. *Nutrition*, *19*, 549–552.

Hermodson, M., & Mahoney, W. C. (1983). Separation of peptides by reversed-phase high-performance liquid chromatography. *Methods in Enzymology*, *91*, 352–359.

Higgins, J. D. G., Thompson, D., & Gidson, T. J. (1996). Using CLUSTAL for multiple sequence alignments. *Methods in Enzymology*, *266*, 83–402.

Hoare, D. G., & Koshland, D. E., Jr. (1967). A method for the quantitative modification and estimation of carboxylic acid groups in proteins. *Journal of Biological Chemistry*, *10*, 2447–2453.

Imoto, T. K., & Yagishita, A. (1971). Simple activity measurement of lysozyme. *Agricultural and Biological Chemistry*, *33*, 1154–1157.

Jedrzejas, M. J. (2000). Structural and functional comparison of polysaccharide-degrading enzymes. *Critical Reviews in Biochemistry and Molecular Biology*, *35*, 221–251.

Kamatari, Y. O., Dobson, C. M., & Konno, T. (2003). Structural dissection of alkaline denatured pepsin. *Protein Science*, *12*, 717–724.

Kanade, S. R., Paul, B., Appu rao, A. G., & Gowda, L. R. (2006). The conformational state of polyphenol oxidase from field bean (*Dolichos lablab*) upon SDS and acid-pH activation. *Biochemical Journal*, *395*, 551–562.

Kokol, V. (2002). Interactions between polysaccharide polymer thickener and bifunctional bireactive dye in the presence of nonionic surfactants. Part 1: Surface tension and rheological behavior of different polysaccharide solutions. *Carbohydrate Polymer*, *50*, 227–236.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*, 680–685.

Liu, Y. N., Lai, Y. T., Chou, W. I., Chang, M. D., & Lyu, P. C. (2007). Structure of family 21 carbohydrate-binding module from *Rhizopus oryzae* glucoamylase. *Biochemical Journal*, *403*, 21–30.

Lopez, M. D., Lopez, F. J., Lopez, A. J., Carreno, F. L., & Toro, M. A. N. (1998). Characterization of fish acid proteases by substrate-gel electrophoresis. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *121*, 369–377.

Mahoney, W. C., & Hermodson, M. (1980). Separation of large denatured peptides by reverse phase high performance liquid chromatography: Trifluoroacetic acid as a peptide solvent. *Journal of Biological Chemistry*, *255*, 11199–11203.

Matsudaira, P. T. (1989). In P. T. Matsudaira (Ed.), *A practical guide to peptide and protein purification for microsequencing* (pp. 53–58). New York: Academic Press.

Monu, P., Prasanna Kumari, N. K., Vikash Kumar, D., Pinky, T., & Jagannadham, M. V. (2009). Stability and unfolding studies on alkaline denatured state (I_p) of pepsin. *Process Biochemistry*, *44*, 906–911.

Nyvall, P., Pedersen, M., Kenne, L., & Gacesa, P. (2000). Enzyme kinetics and chemical modification of α-1,4-glucan lyase from *Gracilariopsis* sp. *Phytochemistry*, *54*, 139–145.

Ohgushi, M., & Wada, A. (1983). Molten-globule state: A compact form of globular proteins with mobile side-chains. *FEBS Letters*, *164*, 21–24.

Pantaleon, D., Yalpani, M., & Scollar, M. (1992). Unusual susceptibility of chitosan to enzymic hydrolysis. *Carbohydrate Research*, *237*, 325–332.

Perrakis, A. (1994). Crystal structure of a bacterial chitinase at 2.3 Å resolution. *Structure*, *12*, 1169–1180.

Roncal, T., Oviedo, A., Lopez de Armentia, I., Fernandez, L., & Villaran, M. C. (2007). High yield production of monomer-free chitosan oligosaccharides by pepsin catalyzed hydrolysis of a high deacetylation degree chitosan. *Carbohydrate Research*, *342*, 2750–2756.

Sawardekar, J. S., Sloneker, L. S., & Jeanes, A. (1965). Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography. *Analytical Chemistry*, *37*, 1602–1604.

Schlamowitz, M., & Peterson, L. U. (1959). Studies on the optimum pH for the action of pepsin on native and denatured bovine serum albumin and bovine hemoglobin. *Journal of Biological Chemistry*, *234*, 3137–3145.

Schuettelkopf, A. W., & van Aalten, D. M. F. (2004). PRODRG – a tool for high-throughput crystallography of protein–ligand complexes. *Acta Crystallography*, *60*, 1355–1363.

Sepulveda, T. P. (1973). Amino acid sequence of porcine pepsin. *PNAS, USA*, *70*, 3437–3439.

Shobha, M. S., Vishu Kumar, A. B., Tharanathan, R. N., Rathna, K., & Gaonkar, A. (2005). Modification of guar galactomannan with the aid of *Aspergillus niger* pectinase. *Carbohydrate Polymer*, *62*, 267–273.

Shobha, M. S., & Tharanathan, R. N. (2008). Non-specific activity of *Bacillus acidopullulyticus* pullulanase on debranching of guar galactomannan. *Journal of Agricultural and Food Chemistry*, *56*, 10858–10864.

Terwisscha van Scheltinga, A. C., Lalk, K. H., Beintema, J. J., & Dijkstra, B. W. (1994). Crystal structure of hevamine, a plant defense protein with chitinase and lysozyme activity and its complex with an inhibitor. *Structure*, *12*, 1181–1189.

Vishu Kumar, A. B., Gowda, L. R., & Tharanathan, R. N. (2004). Non-specific depolymerization of chitosan by pronase and characterization of the resultant products. *European Journal of Biochemistry*, *271*, 713–723.

Vishu Kumar, A. B., Varadaraj, M. C., Gowda, L. R., & Tharanathan, R. N. (2007). Low molecular weight chitosans – preparation with the aid of pronase, characterization and their bactericidal activity towards *Bacillus cereus* and *Escherichia coli*. *Biochimica et Biophysica Acta*, *1770*, 495–505.

Vishu Kumar, A. B., Varadaraj, M. C., & Tharanathan, R. N. (2007). Low molecular weight chitosan-preparation with the aid of pepsin, characterization, and its bactericidal activity. *Biomacromolecules*, *8*, 566–572.

Weaver, L. H., Grutter, M. G., & Matthews, B. W. (1995). The refined structure of goose lysozyme and its complex with a bound trisaccharide show that the goose type lysozymes lack a catalytic aspartate residue. *Journal of Molecular Biology*, *10*, 54–68.