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Digestion of Allergenic Fragments of Gliadin by Purified Enzymes from Hepatopancreas of *Palinurus Elephas* (Fabricius, 1787)

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Viscera from marine organisms are rich potential sources of various enzymes that may have some unique properties of interest for both basic research and industrial applications. Main digestive proteinases detected in the hepatopancreas of marine organism are *trypsin* and *chymotrypsin* (Kishimura H et al., Food Chemistry, 2006). High activity of fish enzymes at low temperatures is interesting for industrial applications, especially in certain food processing. One application is the hydrolysis of protein resistant to gastrointestinal digestion like Coeliac disease. In this work enzymes extracted and partially purified proteases from hepatopancreas from *Palinurus elephas* are analyzed. Moreover, using human Celiac Serum in immunoblotting analyses, we observed that immunological epitope of gliadin, normally recognized from IgE immunoglobulin, disappear after digestion after digestion of gliadin with purified enzyme. These data strongly suggest that, in the partially purified enzymes by hepatopancreas, are present specific proline enzymes.

1 Introduction

Celiac Sprue is an autoimmune disease of small intestine caused by ingestion of gluten proteins from widely prevalent food source such as wheat and barley. The gluten proteins are formed of gliadins, a heterogeneous mix of monomeric proteins that are associated with extensibility properties, and glutenins, a group of proteins that confer elasticity properties and form one of the largest polymers known in nature. Gliadins have highly repetitive sequences with an abundance of glutamine and proline residues. The gliadins have molecular weights from about 28,000 to 55,000 Dalton and are separated into alpha, gamma and omega subgroups. Many studies have attempted to reduce the allergenicity of wheat and milk products. Recently has been proposed that a method for producing hypoallergenic wheat flour enzymatically modified that successfully reduced the wheat allergenicity (Villad´oniga et al., 2007; Watanabe et al., 2000). Proteases have different applications in a wide variety of industries such as detergent, food, and pharmaceutical (Gupta et al.2005). Proteases are mainly derived from animal, plant and microbial sources. Fish viscera are indeed a rich source of digestive enzymes, such as pepsin and the serine proteases, trypsin and chymotrypsin (Gudmundsdóttir, et al. 2005). The property of the digestive enzymes from marine organisms to maintain their activity at low temperature might be very useful in food processing in order to avoid bacterial contamination and unwanted chemical reactions. Early examples of the enzyme family include fiddler crab collagenase I, crayfish trypsin, and shrimp trypsin (Gates & Travis 1969). Other serine proteases were isolated from krill (Turkiewicz et al. 1991), crabs (Grant et al. 1983), crayfish (Klimova & Chebotarev 1999). Most recently collagenolytic serine protease and trypsin from king crab (Paralithodes camtschaticus 1994). The goal of this work is purified specific proline enzymes from hepatopancreas of to be use for food processing applications. We select enzymes able to digest gliadin

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which is proline rich protein responsible to allergenic in celiac patient. For this reason we use serum from celiac patient for immunoblot experiment for investigate reduction of allergenicity of gliadin inducted by hydrolyses.

2 Experiments

2.1 Preparation of enzyme extract

P. elephs were obtained from the mesocosm of IAMC-CNR. The internal organs were separated and only hepatopancreases collected were washed with Phosphate buffered Saline (PBS); than hepatopancreases (50 gr each) were homogenized with 100 ml of extraction buffer (Tris 20 mM HCl, pH 7,5). The mix was centrifuged twice at 10.000 rpm 30min at 4°C. The pellet was discarded and the supernatant was collected and used as the crude protease extract. Crude extract was subjected to ammonium sulphate fractionation (25%, 50%,70% and 90%) for partially protein separation protein. After centrifugation the fractions were resuspended and dialyzed against PBS.

2.2 Digestion of Gliadin at 4°C

In order to quantified the digestion process we used the ninhydrin colorimetric method (Moore, S. and Stein, W.H. 1948). The test tubes containing 25 mg of gliadin (Sigma) each mixed in 5.0 ml of 0.05 M TES buffer and incubate at 37 °C for 15 min. We start the reaction by adding 0.1 ml of enzyme dilution to appropriate tubes. After 5 hs enzyme reaction was stopped by the addition of 0.5 ml of 50 % trichloroacetic acid. After centrifugation at 10.000 rpm for 15 min., 0.2 ml of solution was transferred into test tubes containing 1.0 mL of ninhydrin-citric acid mixture (Moore, S. and Stein, W.H. 1948). Together to to an enzymatic blank reaction. They were Heated for 20 min to 100°C. After cooling, dilute with 5 ml of 50 % n-propanol solution. Let stand for 15 min and read absorbance at 600 nm. From an L-leucine standard curve determine micromoles amino acid equivalent to leucine liberated. One unit liberates peptides from gliadin equivalent in ninhydrin color to 1.0 micromole of leucine in 5 hours at pH 7.4 at 37°C in the presence of calcium ions. To evaluated the activity enzymes on gliadin at low temperature, we incubated 100 microliter of 0,1% gliadin with 10 microgram of different ammonium sulphate fraction of enzyme 24 hrs at 4°. After treatment The reaction was stopped by addition Sample buffer and boiled. Samples were analyzed in SDS page to visualized digestion.

2.3 Electrophoresis and zymography analyses

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to defined purity and molecular weight of the purified enzyme, as described by Laemmli (1970). For zymography analyses the different fraction were mixed in an electrophoresis buffer devoid of reducing agents (2β-mercaptoethanol or the like); the samples also were not subjected to boiling in order to avoid the loss of enzyme activity. The samples thus prepared were stratified in polyacrylamide gel containing gliadin at a concentration of 1 mg/ml. After the electrophoresis separation the gels were washed with a 2 % TRITON X-100 solution in H2O containing 0.02 % NaN3 (3 times for 20 min for each). They were then incubated overnight at 4°C and then dyed with H2Oacetic acid-methanol solution in the ratios 5:1:5 containing 0.8 % Coomassie Brilliant Blue R-250 for 3 h with stirring. The excess dye was removed using a 5 % CH3COOH solution.

2.4 Western Blotting

After 15% SDS-PAGE electrophoresis gel were cast in support and proteins was blotted onto a nitrocellulose membrane (Hybond; Amersham Biosciences; membrane were saturated with 2% nonfat dry milk in Tris Buffer Saline 50mM pH 7.9/Tween 0.05% (TBS-T). After 3 washes in TBST, the nitrocellulose membranes were incubated for two hrs at room temperature, with Human serum from Celiac patient 1 : 500. The primary antibody was followed by peroxidase-conjugated anti Human IgE (1:10000) for 1 hour at r. t.. Immunocomplexes were visualized with the ECL Western blotting kit (Amersham Biosciences) using Hyperfilm.

3 Results

To partially purified enzymes from Palinurus elephas, the hepatopancreas was homogenized to prepare a crude extract. After centrifugation to eliminate non soluble proteins, the supernatant was subjected to ammonium sulphate fractionation (25%, 50% and 70%, 80%) and the different samples were visualized by SDS-PAGE (Figure 1).

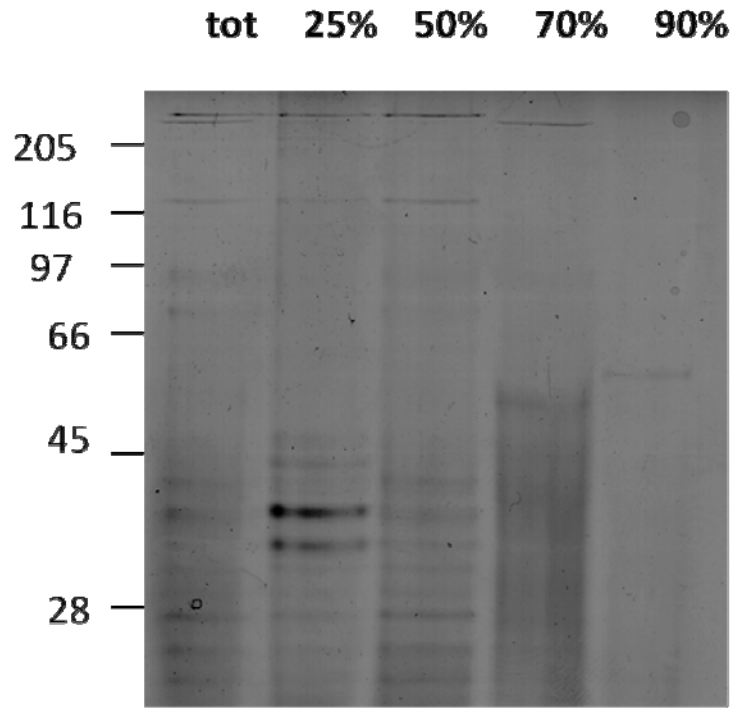

Figure 1. SDS-PAGE analyses of ammonium sulphate fraction : total extract , 25%, 50% and 70%, 90%.In each line were loaded 20 microgram of proteins

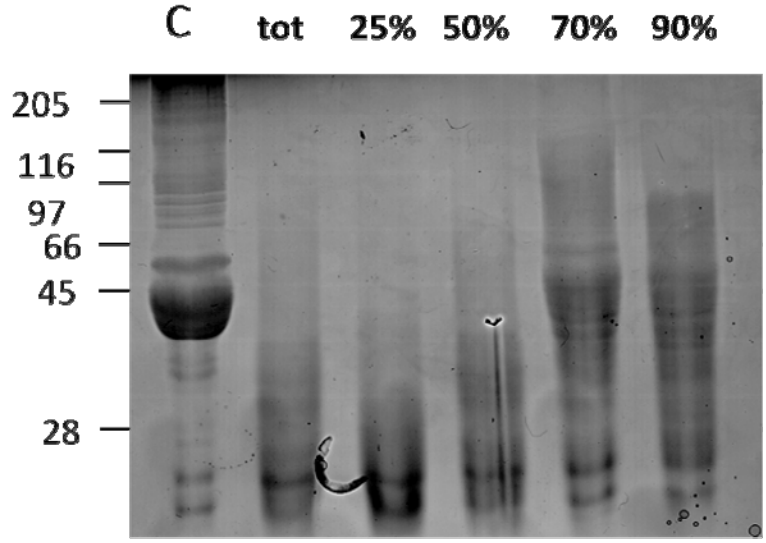

Figure 2: SDS-PAGE analyses C: gliadin control. Gliadin digestion with total extract ,25%, 50% 70%, 90%.

After separation of protein each fraction were tested for ability of digest gliadin substrate. For this propose, we use different methods to identify enzymatic activities. In the first method we select the best fractions in term of capability to digest solution in solution at 4 °C After digestion the gliadin was separated in SDS page to visualize the gliadin undigested and the fragment generated (Figure 2). In the control it is possible to observe the classical protein pattern of gliadin composed by different molecular weights from about 28,000 to 55,000. Moreover we analyze the peptide obtained after enzymatic digestion using ninhydrin assay. The best fraction in terms of activity was the 25% corresponding to 0,354 unit/mg. Both methods have shown that the highest enzymatic activities fraction were present in the fraction obtained by an ammonium sulfate precipitation to 25%. To obtain more information about the molecular weight of enzymes present in the 25% fraction we used zimography enzymatic approach and as shows in (figure 3)

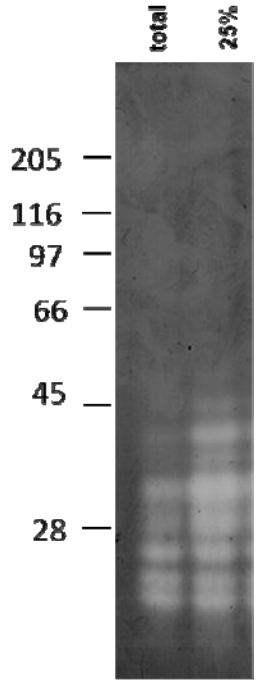

Figure 3: Gliadin zimography (SDS Page at 12%) crude extract (Total); 25% protein fraction (25%)

the presence of several enzymatic activities at low molecular weight range were detected. Moreover the intensity of proteolitic band in the 25% fraction is higher compared with crude extract.

To evaluate the capability of partially purified enzyme to reduce the allergenicity of gliadin we compared the digested and not digested gliadin components in western blot analyze using serum from celiac patients. As show in Figure 5, the IgE present in the serum of celiac patient strongly react a single band of gliadin; while no reaction band was detected after gliading digestion performed with fraction 25% containing enzyme. Consequently, the most allergenic components seem to be completely digested by the action of enzymes present in the 25% fraction.

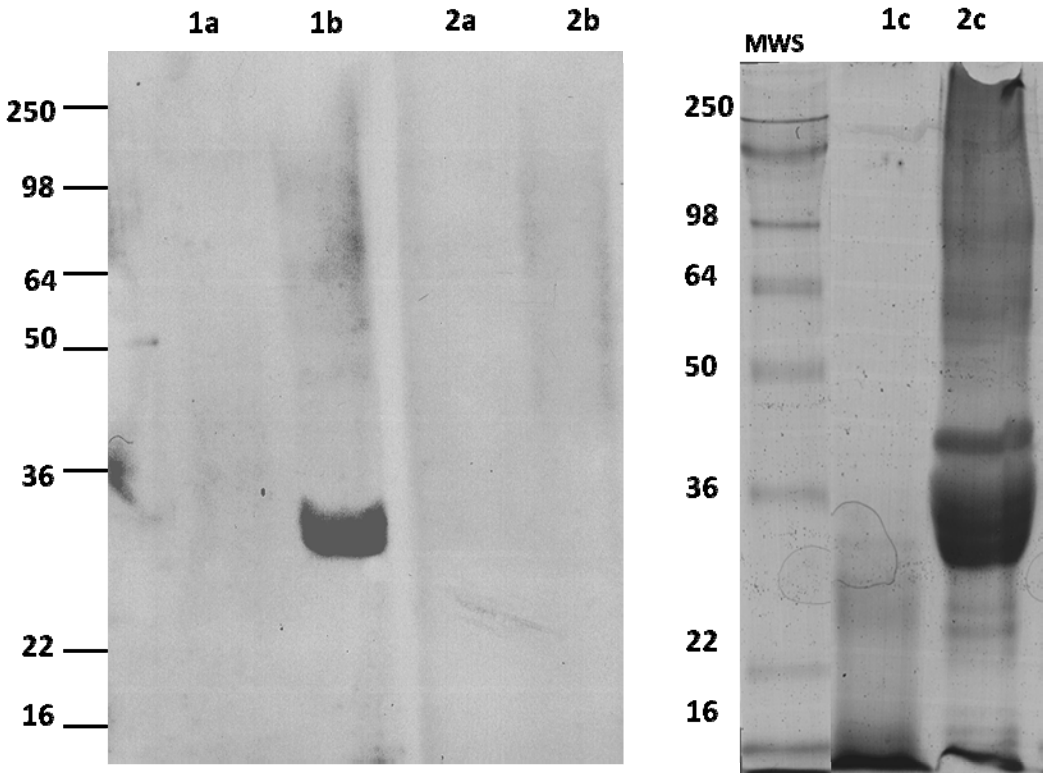

Figure 4: Western blot analyses(1 and 2) of (a) digested gliadin (b) undigested gliadin, using serum from celiac patient (1) or from normal person (2), as control. In (c) SDS PAGE commassie Staining.

4 Conclusion

The use of cold-adapted enzymes in the food industry are several. For example, in the milk industry. Many studies have attempted to reduce the allergenicity of wheat and milk products. We proposed that a method for producing hypoallergenic wheat flour enzymatically modified by marin enzyme can successfully reduced the wheat allergenicity

Our data suggest, that in the partially purified enzymes extracted from hepatopancreas of from *Palinurus elefans*, are presence enzyme able to digest gliadin.. Using immunoblot analysis we observe that wheat allergens are recognized by the IgE antibodies of the sera of a allergic patients. Those allergen after digestion of gliadin with the enzyme was completely digested and that the fragment do not react with The IgE; a similar results has been show by P. Phromraksa et al. (2008). at present, experiments are in progress to identify the marine prolyl endopeptidases and/or prolyl oligopeptidases, a family of serine proteases having the ability to hydrolyse the peptide bond on the carboxyl side of a proline residue. With the new molecular biology technology will be possible to clone single genes and to produce marine organism enzymes by in vitro synthesis systems. For example, enzyme with collagenolitic activity are synthesized from Paralithodes camtschaticus and cystein proteases cathepsin has been produced from Metapenaeus ensis.

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