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(Research Article)

Degradation of gluten using plant proteases

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Abstract

The paper describes the fact of the presence of a proteolytic enzyme in the leaves of the natural hybrid variety of grape - Isabella (Vitis Labrusca L.) and the perspective of its practical application for breaking down wheat gluten. As our research has shown, this enzyme effectively degradates the constituent components of the protein fraction of wheat flour-the gluten fraction. It is known that the wheat gluten is a strong allergen for many people, which can initiate a disease such as celiac disease. Protease from Isabella leaves carries out deep hydrolysis of gluten to low molecular weight soluble peptides and amino acids. This effect can be successfully used in the production of gluten-free bread products.

Keywords: Enzyme; Protease; Hydrolysis Of The Gluten; Electrophoresis; Gluten-Free Fraction

1. Introduction

Bread and bakery products play a large role in the human diet. Bread made from wheat flour contains 34% carbohydrates, 34% protein, 24% dietary fiber, and 13-32% minerals and vitamins, almost fully meeting the daily recommended requirement. In addition, biologically active components of wheat include dietary fibers, phytochemicals such as carotenoids and polyphenols, phenolic acids, flavonoids and lignans, and vitamins. [1,2] At the same time, wheat grains (rye, barley, wheat) contain proteins rich in proline and glutamine-prolamins, the consumption of which in some people can initiate gluten-dependent diseases such as celiac disease, wheat allergy, asthma, anaphylaxis [3,4].

Proline and glutamine determine the structure of wheat protein, which the enzymes of the human digestive tract cannot completely break down. As a result of improper fermentation, the so-called Gluten peptides, which contain 10% gluten taken with food. Insoluble gluten peptides are often absorbed by the lining of the small intestine and activate immune cells in the gut in people with celiac disease. This type of activation is not observed in healthy people - insoluble peptide fragments are excreted together with, for example, urine.

Celiac disease (hereditary gluten enteropathy) is a chronic multifactorial inflammatory disease, which is characterized by intestinal and non-intestinal manifestations - production of specific blood serum antibodies and autoimmune response, lesions of the small intestine mucosa, etc. [5,6]

Celiac disease is currently the only autoimmune disease that can be cured. Patients are successfully managed by following a gluten-free diet throughout their lives [7,8]. Although the solution to the problem seems simple at first glance, we must pay attention to such factors as the hidden contamination of food products with gluten. Some food products contain traces of gluten: ice cream, yogurt, milk powder, cream, condensed milk, cheese, mayonnaise, and margarine. The demand for gluten-free products is increasing, but it should also be noted that in certain cases people choose gluten-free products for self-insurance and not because they have a medical indication - for example, celiac disease, intestinal failure syndrome, etc.

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Sometimes people feel better by switching to a gluten-free diet because they eliminate or drastically reduce the consumption of problematic products such as beer, so-called "snacks" and other products. These are products that are often associated with irritable bowel and related digestion problems. While a gluten-free diet may reduce certain symptoms in some people, it may lead to malnutrition in others because vitamin B12, folic acid, zinc, magnesium, calcium, and selenium are significantly reduced in the diet, as well as calcium. All of these will affect the gut microbiome, and it is possible to have far-reaching negative consequences. Often gluten-free products are high in calories due to the complex chemical additives that must be added to achieve the texture and consistency that gluten provides. Recent studies have shown that steady consumption of gluten-free pasta products leads to a sharper increase in blood glucose levels than consumption of pasta made from regular wheat flour. Most likely, this is explained by the fact that the texture of wheat is imitated by highly refined carbohydrates. Therefore, we should not be surprised that their consumption dramatically increases the production of sugar in the body, that is, in the long term, there is an increase in weight and a high risk of developing diabetes

Thus, it is important to find a technological way through which a functional group of consumers will receive a gluten-free product without various unwanted additives, which will preserve the desired taste and other properties.

Traditionally, gluten-free bread products for people with celiac disease are made from raw materials that do not contain or contain very little gluten, such as buckwheat, rice, potato or corn starch. There are reports about the use of flour obtained from hemp leaves and seeds and fruits of the Mayday tree (bird cherry) [9]. It must be said that in each such case, additional efforts were needed to preserve the natural texture, taste properties and other parameters.

Among the many ways to solve the problem, two of them attract attention -

- Intestinal digestion of gluten by glutenases obtained from different sources - bacteria and fungi (*Aspergillus niger, Flavobacterium meningosepticum, Sphingomonas capsulate, Actinoallomurus, Streptomyces lividans*), plants (barley), engineered recombinant proteins with different biological activities are described in vitro (Lactilglutenase - IMGX003) [10].
- Pretreatment of gluten with bacterial/fungal/plant/synthetic endopeptidases. Wheat flour or flour products may be modified by fermentation by bacteria or fungi. These organisms secrete proteolytic enzymes, with which gluten becomes less toxic as a result of processing

It is in this direction, that is, the possibility of breaking down gluten into small peptides and amino acid components through proteolytic enzymes, that attracted our attention.

Proteases are enzymes that catalyze the hydrolysis of other proteins and form the largest group of enzymes, accounting for approximately 2% of the human genome. Due to structural and functional diversity and several mechanical features, proteases perform various functions, including digestion of intracellular proteins and nutrients, strengthening of the immune system. [11]

The flora of Georgia is distinguished by the wealth of medicinal and food plants, most of them attract attention with their chemical composition. We focused our attention on Isabella (Vitis Labrusca L,), which is a hybrid cultural plant. It is distinguished by its resistance to various diseases or agricultural pests, the fruit is characterized by good taste, although the leaf has not yet been widely used.

When we studied the biological properties of Isabella leaves, the presence of proteolytic enzymes was determined in them. It should be noted that grape leaves are a waste product of the agricultural industry, which is collected in large quantities and so far their utilization is difficult and expencive. Considering the cheapness of raw materials, our task was to use the biological properties of these leaves in various fields. One such direction has been the breakdown of gluten in the production of bakery products to avoid the effects of diseases such as celiac disease.

Due to the fact that gluten-free bread is made from gluten-free materials, the product loses the taste properties of bread. That is why we tried to remove gluten from traditional bread through proteolytic enzymes and preserve its taste properties.

Based on the above, we aimed to obtain proteins with protease activity from the raw materials we selected for their further use.

2. Materials and methods

2.1. Extraction of proteolytic enzyme

In order to extract proteins with proteolytic activity, the raw material (leaves of Isabella) is finely chopped in a porcelain mortar or extractor, 0.1M phosphate buffer (PBS pH=7.2) is added in a ratio of 1/10 (mass/volume). Homogenization takes place at a temperature of 22^0 C until a homogeneous mass is obtained. The obtained material is filtered and centrifuged at 3000 rpm for 10 minutes and collected supernatant.

2.2. Determination of proteolytic activity

Protease activity is determined in the collected supernatant [12]. During incubation with an insoluble substrate, the action of proteases in the reaction area starts to break down the substrate, peptide fragments and amino acids appear in the reaction area - there is an increase in peptides, which is recorded spectrophotometrically (λ=660 nm) using a UV–VIS spectrophotometer (Perkin Elmer, USA) against a reagent blank. In our case, for each sample, blank value was taken 30 minute incubated substrate samples without enzyme. After cooling samples we added Enzyme and immediately measure with Folin and Ciocalteu reagent. This type of blanks was taken to exclude enzyme and substrate influence on absorption.

2.3. Gluten extraction

An elastic dough is prepared to obtain gluten. In order to remove the starch, soluble proteins and other accompanying substances in the flour, the dough is washed in running water before the color of the water becomes clear.

2.4. Breakdown of gluten

In order to proteolytically break down gluten, an enzyme solution is added to gluten in a ratio of 1/10 (mass/volume) and incubated at a temperature of 27^0 C. The degree of transformation of the substrate is assessed both qualitatively (decreasing the gluten-containing mass and reducing its size) and quantitatively (by determining the protein by Lowry's method) [13]

2.5. Electrophoresis

Disk electrophoresis is performed in a 12% polyacrylamide gel under denaturing conditions in the presence of SDS. [14,15,16]

3. Results and discussion

Our research has shown presence of proteolytic enzyme in Isabella leaves(Vitis Labrusca L.). Some characteristics of this proteolytic enzyme preparation obtained by us are presented in the table 1.

Table 1 Parameters of protease from Isabella leaves

Source of enzyme	A U/100	protein mg/ml	U/ml	Total U/ml*V	U/mg	mg.prot/total prot	Total Activity
Isabella leaves	0.00285	0.043	0.143	25.65	0.33	77.4	25.65

It is clear,that we have obtained the enzyme preparation,which has a high proteolytic activity.

Our research has shown that Isabella Protease effectively degradates gluten. For this purpose, we removed the gluten by simply washing the dough prepared from wheat flour under running cold water. The gluten obtained in this way was treated with a protease preparation obtained from Isabella leaves.

As mentioned above, we have added Isabella enzyme solution to gluten in a ratio of 1/10 (mass/volume), so the proteolytic activity in reactor was 1.43 U/ml per 1 g. of gluten. Under the influence of protease, gluten, as an insoluble protein substance of the dough, was quickly and efficiently broken down into soluble peptide fragments. The rate of protein accumulation in the reaction area was determined by Lowry's method (see Table 1).

Table 2 Protein accumulation rate in the reactor before and after treatment with Isabella gluten protease

Parameter	Rate (mg/ml)
Protein concentration in the reactor before hydrolysis	19,420
Protein concentration in the reactor after hydrolysis	31,0611

Kinetic accumulation of protein over time in the reactor is shown in figure 1. (To calculate the protein concentration, we use the corresponding calibration curve.)

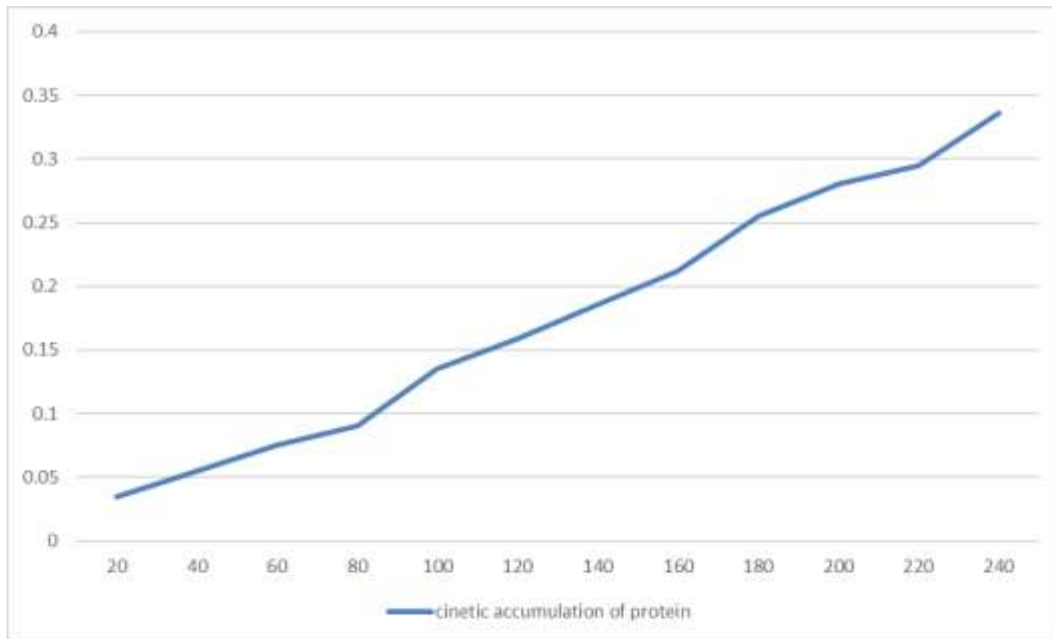
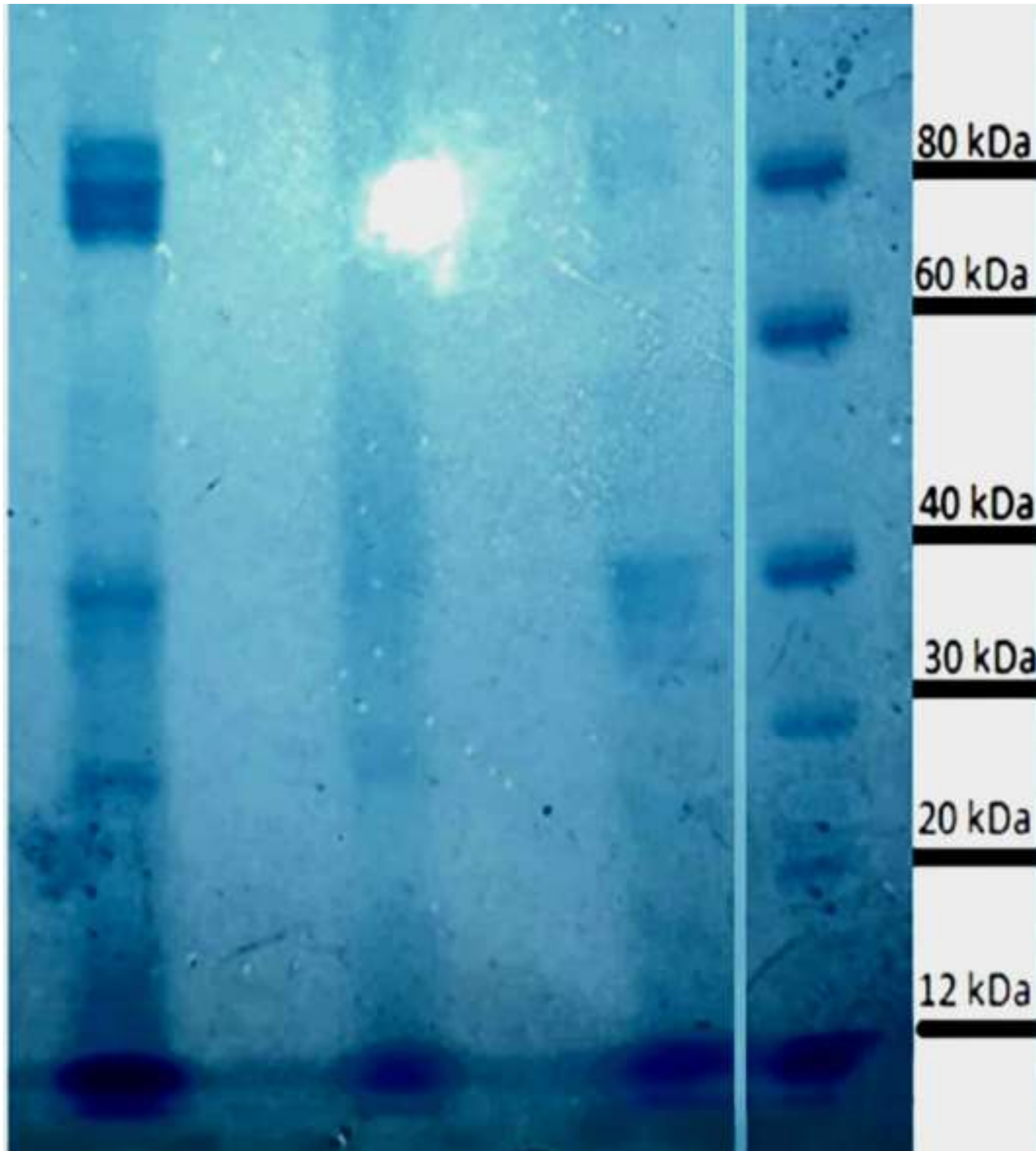


Figure 1. Changes in protein accumulation in the reaction area over time (time in minutes on the abscissa axis, amount of protein (mg/ml) on the ordinate axis)

The increased concentration of protein in the reaction area ($\Delta C \approx 11.64$ mg/ml) indicates that the insoluble gluten is transferred to a soluble state after enzymatic impact (hydrolysis). The question is what size polypeptide fragments are formed as a result of hydrolysis.

It is possible that high-molecular fragments of gluten, even if soluble, also cause an allergenic effect. As the authors claim [5,6], these are mainly insoluble peptides to a greater or lesser degree. Accordingly, the lower the molecular fractions of gluten, the greater the probability of avoiding its allergenic effect considering the fact that our hydrolyzate is completely and well soluble in water. In our opinion, the breakdown of gluten by the addition of protease and the increase of protein in the reaction area was not a complete indicator, it remained unclear how deeply the protease cleaved gluten, the complex of which consists of various proteins [17].

In order to answer this question, disk electrophoresis in 12% polyacrylamide gel was performed. (SDS-PAGE). Both the gluten itself and the preparation containing Isabella's protease, as well as its hydrolyzate, were taken. (see Figure 2).



1 2 3 4

Figure 2. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE). Lane 1 – electrophoresis of gluten under denatured conditions, Lane 2 – Sample of gluten treated with Isabella protease; Lane 3 – Electrophoresis of isabella enzyme-containing extract under denaturing conditions; Lane 4 – molecular mass markers.

As can be seen from the electrophorogram, Isabella's proteolytic enzyme, with its specificity, deeply hydrolyzes all gluten fractions and leads it to final products - low-molecular peptides and amino acids, which is indicated by the disappearance of gluten fragments on the hydrolyzate paths.(Lane 2).

The mentioned facts allow us to assume that it is possible to develop a technology for the production of gluten-free bread products. It is important that today gluten-free bread is made from raw materials that do not contain gluten, e.g. Buckwheat, potatoes, rice and others. The taste properties of products made from these raw materials are different from bread made from wheat flour. That is to say, there is a demand for such gluten-free bread, which will have the taste properties of bread made from wheat flour. It is the way that we propose that allows us to develop the production technology of gluten-free bread from Isabella (Vitis). Labrusca L,) using proteases.

4. Conclusion

We have studied the physico-chemical properties of Isabella leaves and detected the presence of protease in the extract of leaves.

Also,we have detected the breakdown of gluten under the influence of Isabella protease. This effect can be successfully used in the food industry for the purpose of removing gluten in bread, in order to provide the appropriate target group with a product that would satisfy both taste and other characteristics, e.g. Texture, composition, etc.

It is promising to use Isabella's proteolytic enzyme to produce other types of gluten-free products, such as beer, cookies and pasta, confectionery and others.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed

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