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Research Article

Emmer starch isolation by alkaline protease digestion of wet-milled seeds

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Abstract

In today's dynamic world, it is necessary to explore more alternative options for starch isolation and develop technologies to obtain better quality products. The presence of impurities in the composition of starch is an indicator that affects its properties. Starch was isolated from whole emmer seeds. The present study aims to compare the protein content of isolated emmer starch obtained by a standard alkaline method and a combined alkaline with an enzymatic method. The conducted method for the isolation of starch from emmer was with a duration of the alkaline treatment of 24 and 48 h. The results were compared with the results obtained from the combination of alkaline (24 h) and enzyme (with an amount of enzyme 0.05-2.2% for 1-8 h) treatment. The results showed that the long alkaline treatment could be replaced by a shorter one with 0.05% enzyme for 1 h. The lowest values of protein in the composition of starch were reported after treatment with 1.1% multienzyme preparation for 8 h.

Keywords

emmer seeds, alkaline starch isolation, multi-enzyme complex, protein content

Abbreviations

ANOVA – analysis of variance; SD – standard deviation

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Introduction

In order to increase the diversity of raw materials, poorly studied crops are now being analyzed for potential use in food processing. Emmer, obtained by crossing the intersection of the wild species of *Triticum dicoccoides* (Schrank) Schűbler and *Triticum durum* Desf. is a type of tetraploid wheat. Emmer wheat originated from the Middle East and it is widely spread in the Mediterranean basin. It was among the earliest domesticated *Triticae* by humankind and the principal wheat of old–world agriculture in the neolithic and early bronze ages (Suchowilska et al. 2012; Weide 2015; Békés et al. 2017; Fadida-Myers et al. 2022). Today emmer wheat is a minor cereal. However, the increasing attention to sustainable agriculture and the demand for organic foods has raised the interest in emmer wheat (Biradar et al. 2022). The attention towards the ancient species has also been renewed by the increasing demand for traditional products, the request for species suitable to be grown in marginal areas and the need to preserve genetic diversity. Emmer wheat is a rural cereal crop characterized by a higher yield that adapts to soils that are poor in nutrients and is resistant to extreme weather conditions (cold and hot climates), dryness and humidity, which is why it is mainly grown in the hills and low mountain areas. Thus, it has been rediscovered that their better performance in disadvantageous pedo-climatic conditions as compared to modern cultivars. Emmer and einkorn are also considered to be more resistant to diseases than modern wheat. Certain accessions have been identified as resistance sources against fungal diseases, including powdery mildew, stem, yellow and leaf rusts, tan spot, Septoria blotch, bunts, and Fusarium (Zaharieva et al. 2010; Góral and Ochodzki 2017; Bencze et al. 2020; Șerban et al. 2021). Scientists are interested in emmer as a genetic depository for many agronomic and nutritional traits with important commercial issues. It has been recognized as a very healthy cereal and is recommended in the diet of people suffering from allergies, colitis and high blood cholesterol due to its nutritional and functional properties such as its contents of resistant starch, fiber, antioxidant compounds, minerals, and poor in fats (Giacintucci et al. 2014; Arzani 2017).

Starch is known as a biocompatible, biodegradable, non-toxic, eco-friendly and inexpensive natural polysaccharide (Rodrigues and Emeje 2012). Starch is the main carbohydrate synthesized and stored in most plants and plays an essential role in human and animal nutrition. Different types of cereals contain starches that vary in the ratio of amylose and amylopectin and the structure of the starch granules, which determine the physicochemical and functional properties such as cohesiveness, gelatinization, retrogradation and susceptibility to enzymatic degradation (Alcázar-Alay and Meireles 2015; Mohamed 2021). Starch plays a major role in providing energy for human activity and has a broad application in food and non-food industries (Colussi et al. 2014).

Emmer starch has been found to be slowly digestible due to the complexity of the starch structure, high degree of crystallinity and high amylose content (Khuladunga and Simsek 2023). The main properties that make starch widely used are its gelling properties (Ai and Jane 2014)

Research efforts are increasingly focused on the production of wheat for various starch paste applications, seeking desirable properties such as high volume and swelling power, high peak viscosity and low gelatinization temperature. The results show that the swelling process of starch granules is highly dependent on the protein content (Yang et al. 2019).

Since there is no data in the scientific literature on the use of emmer seeds as a raw material for the production of starch on an industrial scale, and no technological scheme for the production of emmer starch has been developed. The present study aims to propose a method for the isolation and purification of emmer starch. An alkaline isolation method with modifications and the use of a multienzyme complex was applied to obtain emmer starch with lower protein content.

Materials and Methods

Materials

Emmer seeds. The emmer seeds (*Triticum dicoccum* /Schrank/ Schübler), cultivar Farro (Italy origin) were collected from the village Kukos (North Greece). After analyzing the chemical composition of spelt grains, the results obtained for the percentage of macronutrients were presented in Table 1 (Petkova et al. 2019).

Table 1. Proximate composition of emmer seeds

Proximate composition	Values, %
Moisture	9.70±0.12
Lipid fraction	1.60±0.01
Protein	17.50±0.25
Starch	67.10±1.20

Multi-enzyme complex (Multi-Protozin). Multi-Protozin (Biovet JSC, Peshtera, Bulgaria) is a multi-enzyme complex with a wide range of enzyme activities, including the enzymes xylanase, *β*-glucanase, *α*-amylase, cellulase, protease, lipase, *etc*. (Table 2).

Table 2. Enzymes activities

Enzymes activities	Minimum activity, UI. g^{-1}
Endo-1,4-*β*-xylanase	250
Endo-1,3(4)-*β*-xylanase	1000
Endo-1,4-*β*-glucanase (cellulase)	350
α-amylase	350
Protease	7500
Lipase	100
Polygalacturonase (pectinase)	2000

Methods

Starch isolation by alkaline digestion. Although emmer is the ancestor of wheat the technology of starch production from wheat cannot be used for the isolation of starch from emmer. The reason is that emmer does not create a gluten structure (Geisslitz et al. 2019; Lacko-Bartosova et al. 2019). Due to the difference in gluten properties of wheat and emmer flour (Belcar et al. 2020) and the close chemical composition of emmer and rice seeds a rice starch production technology was chosen.

Due to the lack in the scientific literature of a specific technological scheme for obtaining starch from emmer a technology for the production of rice starch by alkaline digestion was used with modification (BeMiller and Whistler 2009). The alkaline method of starch production is based on the alkaline dissolution of the protein. The broken rice grains are treated with 0.3-0.5% sodium hydroxide solution (pH range 13.0-13.5) for up to 24 h in the temperature range from room temperature (25±1°C) to 50°C. This causes the grain to soften and the proteins to dissolve. Wet grinding of the soaked grain in sodium hydroxide solution, releases the starch in the form of a suspension, which is stored 10-24 h to further dissolve the proteins. The cell wall material is then removed by filtration, and the starch slurry is washed with water (to remove the protein), neutralized and dried. An advantage to the alkali process is the ease with which the alkali solution allows for modification of the starch since most modification reactions are done at high pH values (BeMiller and Whistler 2009).

Enzyme activity. The enzymatic activity of Multi-Protozin was analyzed to determine the pH and temperature optimum of its proteolytic enzyme. Two ml 2% solution of casein was used as substrate. Two conditions are applied: in the first, the samples stayed 10 min at the same pH - 8 but at different temperatures (35, 45, 55, 65, and 75°C), and in the second - at 35°C but at different pH (7, 8, 9, and 10). It was added a 2 ml enzyme complex and left in a water bath at the appropriate temperature for 15 min. Then, it was added 4 ml of 5% solution of CCl_3COOH was left for another 20 min. After filtering it was taken 1 mL of the filtrate was, adding 1 mL of Folin solution and 5 ml of 0.5 M Na_2CO_3 was. After 30 min the sample was analyzed on a spectrophotometer at a wavelength of 670 nm. The analysis is performed against control (2 ml of casein, 5 ml of CCl_3COOH solution, and 2 ml of enzyme solution are placed in a test tube. After 20 min the control was filtered and the filtrate was developed in the same way as the experimental samples) (Kaverzneva 1971).

Enzymatic treatment. The emmer starch was treated with different amounts of Multi-Protozin (0.05, 0.1, 0.2, 1.1, and 2.2%) for different times (1, 2, 5, and 8 h) to reduce the amount of protein in the starch.

Protein content analysis. The total protein composition described by (AOAC 2016) was analyzed using the Kjeldahl method. It used UDK 152 System (Velp Scientiffica, Italy).

Digital images of starch granules. In order to establish possible disruption of the structure of the starch granules from emmer after alkaline and enzymatic treatment, a microscopic photograph was taken. The digital image was made by a BOECO BM-180 (Germany) microscope equipped with an MDCE-5 USB 2.0 digital camera (China).

Statistical analyze. All results were expressed as the mean ± standard deviation (SD). The data in Table 3. were evaluated statistically using one-way analysis of variance (ANOVA), and Tukey's test ($p < 0.05$) to establish the statistical significance of the differences between the starch samples.

Results and Discussion

Starch isolation by alkaline digestion. The development of food technology and the search for alternative raw materials for starch production were the reasons for choosing emmer seeds for our research.

Taking into account the specificity of the culture used, a partial modification of the already known alkaline isolation of rice starch was necessary (Fig. 1). Unlike the traditional process scheme, whole emmer seeds are used to prevent contamination of the starch with the emmer coat during dry milling, which requires additional purification. This necessitates greater purification and washing of the resulting suspension. Another result of using whole grains is the increased amount of protein obtained. This necessitated additional processing with an enzyme complex. Emmer is soaked with 0.1 N sodium hydroxide in a ratio of 2:1 at 45℃. The temperature regime is chosen so as to intensify the process, but also to prevent starch gelation.

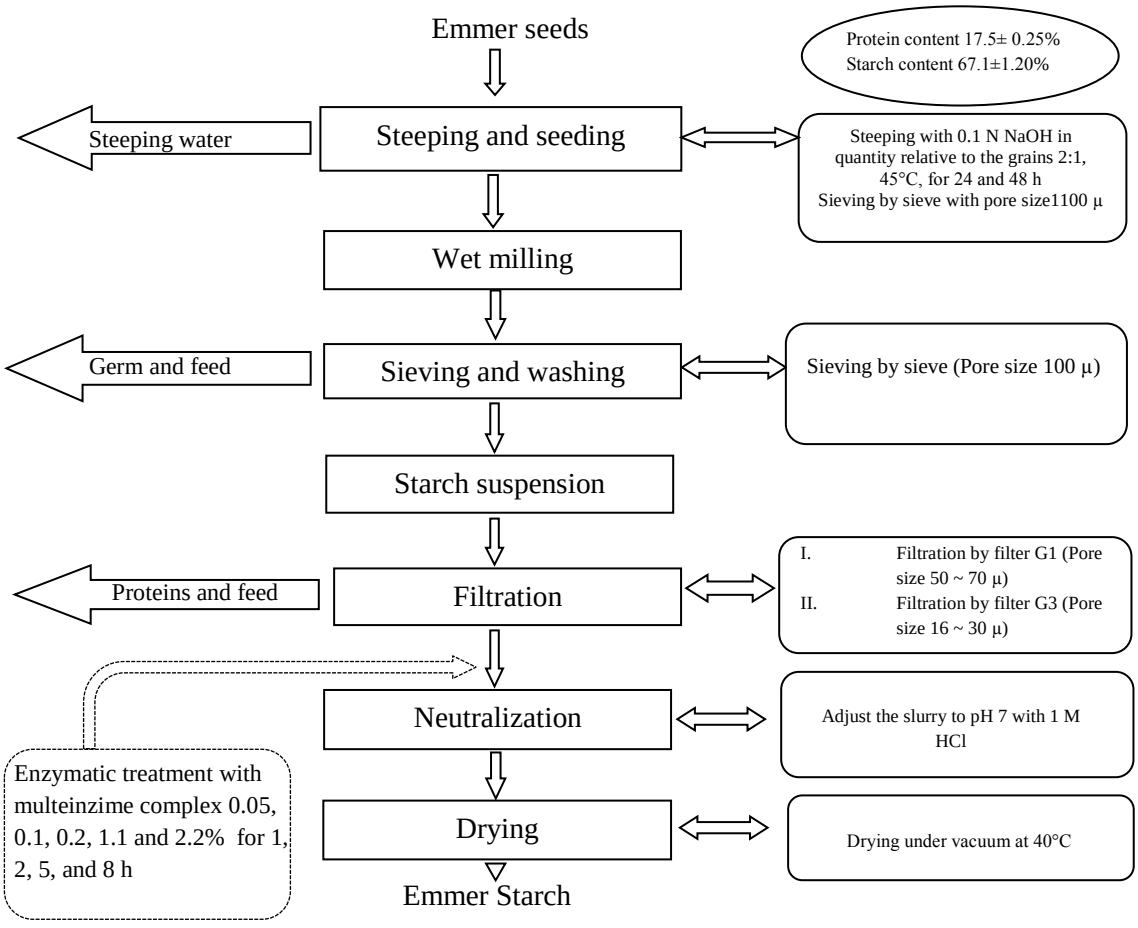


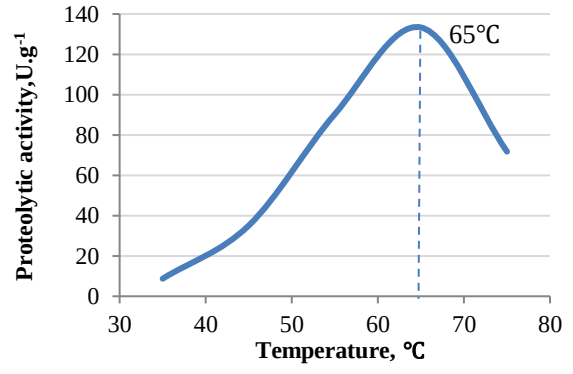
Figure 1. Technology of starch isolation by alkaline digestion.

Alkaline treatment was carried out in addition to a time of 24 h, as per technology, and also for a time of 48 h, in order to compare the results obtained. The solution was separated by passing the beads through an 1100 µm sieve. Emmer seeds were then wet ground, sieved through a 100 µm pore size sieve and washed with clean water to remove germs and shells. Additional filtration was done to remove the bran and proteins. Filtration through 50-70 µm pore size filters is first performed to remove the remaining bran after milling, and subsequent filtration through 16-30 µm pore size filters to pass the dissolved protein by spinning and washing several times with clean water. Neutralization is carried out with 1 M hydrochloric acid to pH 7. The resulting starch is dried under vacuum at 40℃ (a lower temperature regime was chosen to have smaller changes in the starch). The conditions and the temperature regime are selected in such a way as to preserve the integrity of the starch grains (Fig. 1). Various studies have addressed how proteins affect the physicochemical and functional properties of starch, including digestibility and potential applications in the food industry (Wang et al. 2020). Proteins often inhibit gelatinization and limit starch retrogradation. They affect the textural, sensory and digestive properties of many starch-based foods.

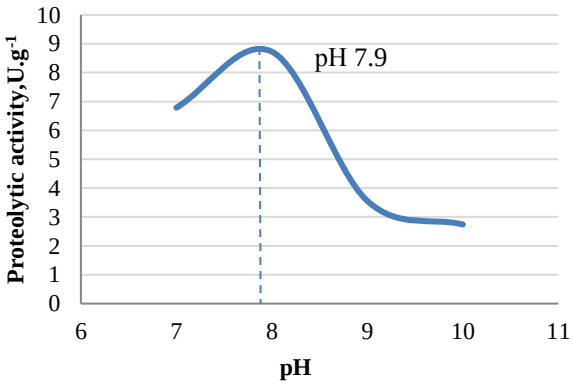
Starches and proteins are two major types of biopolymeric components, especially in many foods consumed worldwide, that provide energy and nutrients needed by the human body (Zhang et al. 2021; Lu et al. 2022).

On the other hand, studies have shown that cell walls, proteins and non-starch polysaccharides prevent the destruction of starch during heat treatment in the presence of water. Additionally, they encapsulate starch granules and create physical barriers to enzyme accessibility (Chi et al. 2022). After the isolation of emmer starch by alkaline treatment for 24 and 48 h, an analysis of the protein content of the starch composition was performed. The results for the protein content of emmer starch after alkaline digestion after 24 h and 48 h are shown in Table 3. These results necessitated further processing of the starch for purification.

Enzyme activity. To purify the obtained starch from proteins and create optimal conditions for the action of the multienzyme complex, its proteolytic activity was analyzed in relation to temperature and pH. A multi-enzyme complex Multi-Protozin was used for additional purification of the starch. Although the multi-enzyme complex has different activities, the highest enzyme activity is proteolytic (Table 2). However, the optimal conditions for its use were not given by the manufacturer. Therefore, the proteolytic enzyme activity was analyzed to find the optimal temperature and pH for its action. The results for optimal temperature and pH are shown in Fig. 2(a) and 2(b), respectively.



(**a**)



(**b**)

Figure 2. Enzyme activity according to temperature (**a**) and pH (**b**)

The results showed that the optimal conditions for proteolytic activity of the multi-enzyme complex were a temperature of 65℃ and a pH of 7.9. The optimal temperature for enzyme activity is higher than the gelatinization temperature of emmer starch (63±1℃) (Zaparenko et al. 2020) and therefore, a temperature of 55°C and pH of 8 was chosen for enzymatic treatment of emmer starch.

Enzymatic treatment. Based on the obtained results regarding the protein content (Table 3) of the isolated starch, it can be concluded which type of treatment is most suitable from the point of view of purification. High protein content would affect starch properties (Yang et al. 2019). With long-term soaking of the raw material (over 24 h), the protein content was reduced. Increasing the steeping time in sodium hydroxide solution resulted in lower starch protein content. Alkaline treatment of starch for 24 h reported a decrease in protein content with 5.12% and after 48 h with 8.35%. On the other hand, when the raw material stays in an alkaline solution for more than 24 h, the starch is modified under the action of the alkaline solution due to the destruction of its intermolecular hydrogen bonds caused by the alkali, which led to changes in its properties (Fan and Picchioni 2020). It can be seen that the alkaline treatment alone does not give good enough results in terms of the amount of protein in the starch composition. This gives reason to choose a shorter alkaline treatment of the emmer seeds, combining it with an enzyme treatment. The following concentration levels 0.05, 0.1, 0.2, 1.1 and 2.2% were selected for enzyme treatment. And the time of action of the enzyme complex is 1, 2, 5 and 8 h, respectively.

Table 3. Protein content in the emmer starch produced by alkali digestion and a combination of alkali digestion and enzyme treatment

Method of isolation	**Time for isolation, h**					
			24 h			
	48 h	Without enzyme treatment	+1h enzyme treatment	+2h enzyme treatment	+5h enzyme treatment	+8h enzyme treatment
Alkaline isolation	$9.15^{a} \pm 0.46$	$12.38^{b} \pm 0.62$	-	-	-	-
Alkaline isolation + 0.05% multienzyme	-	-	$8.77^{a} \pm 0.44$	$7.17^{c} \pm 0.22$	$7.03^{c} \pm 0.14$	$6.22^{d} \pm 0.25$
Alkaline isolation + 0.1% multienzyme	-	-	$7.2^{c} \pm 0.18$	$7.03^{c} \pm 0.35$	$6.20^{d} \pm 0.16$	$6.15^{d} \pm 0.12$
Alkaline isolation + 0.2% multienzyme	-	-	$7.15^{c} \pm 0.36$	$6.80^{c} \pm 0.20$	$5.53^{ej} \pm 0.22$	$5.60^{j} \pm 0.17$
Alkaline isolation + 1.1% multienzyme	-	-	$7.08^{c} \pm 0.28$	$5.84^{dj} \pm 0.23$	$5.10^{f} \pm 0.05$	$4.80^{i} \pm 0.12$
Alkaline isolation + 2.2% multienzyme	-	-	$6.90^{c} \pm 0.35$	$5.40^{e\,j} \pm 0.14$	$5.35^{ef} \pm 0.21$	$5.17^{f} \pm 0.05$

The results showed that enzyme treatment with 0.05% multienzyme complex for 1 h reduced the protein content of starch produced after 24 h of alkaline digestion and the results were similar to the protein content of starch after alkaline treatment for 48 h. These results proved that the two methods can be used interchangeably, thus avoiding the possibility of greater changes in starch properties due to longer alkaline treatment. The largest difference based on the initial amount of protein before enzyme treatment in the range of 30-40% was observed in the first hour (Fig. 3). However, it became clear that at low enzyme concentrations of 0.05 and 0.1%, as the time of starch treatment increased, the decrease in the amount of protein was smoother. The same trend was observed with increasing amounts of enzyme when treated for 1 h. It is observed that when the starch is treated with the enzyme in the range of 0.1-2.2%, more significant changes in the reduction of the amount of protein were shown up to 5 h. However, the lowest protein content of 4.80±0.12 was reported for the starch obtained after alkaline treatment for 24 h and additional enzyme treatment with 1.1% enzyme for 8 h. From the results, it is also evident that the increased amount of enzyme used above 1.1% does not significantly affect the amount of the protein. The results in Table 3. showed that starch treated with enzyme 0.2, 1.1 and 2.2 for 2, 5 and 8 h (except for the sample treated with 0.2% enzyme for 2 h)

had similar levels and relatively low values of protein content. For a more accurate comparison of these results, a Tukey's test ($p<0.05$) was performed and the same results have the same coefficient. These results are also confirmed by the data presented in Fig. 3 for the percentage reduction of proteins relative to the initial amount. Similar results have been reported for the enzymatic treatment of rice starch (Lumdubwong and Seib 2000).

Due to similar results in protein reduction, it would be appropriate to choose a shorter regimen and lower enzyme concentration to save time and be cost-effective (Robinson 2015).

The time factor in the enzymatic treatment at the beginning is more influential. As the processing time progresses, the enzyme action is affected to a lesser extent, so it could not be considered as influencing to the same extent as the amount of enzyme.

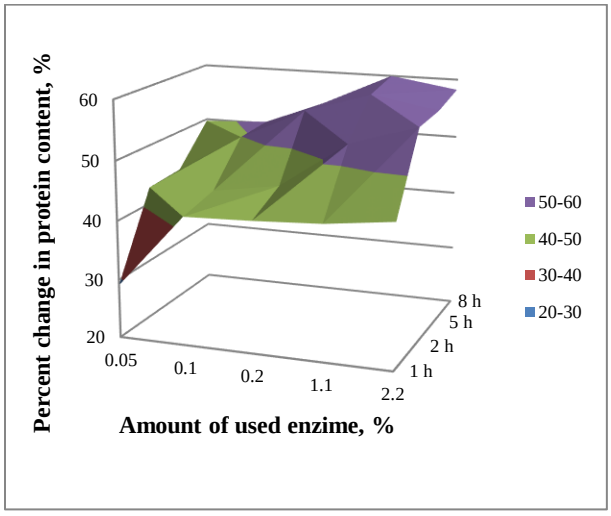


Figure 3. Reduction of protein content in emmer starch, depending on the amount of enzyme and the duration of processing

From the graphically presented data on the percentage change in the amount of protein in the starch composition, it can be seen that during the first hour and at a lower concentration of the enzyme complex, the change was most pronounced. The results presented in Fig. 3 can be used for giving predictive data if another raw material with a different amount of initial protein and the same enzyme complex is used.

From the microscopic picture, it can be seen that there is no violation of the structure. Starch grains of emmer (ancient wheat), like wheat, are oval and lenticular in shape, with the sizes of type A starch granules (10-40 μm) and smaller type B starch granules (<10 μm) (Shang et al. 2023). Despite the presence of α-amylase in the composition of the multienzyme complex, due to the low enzymatic activity per gram compared to protease, it does not affect the structure of starch granules.

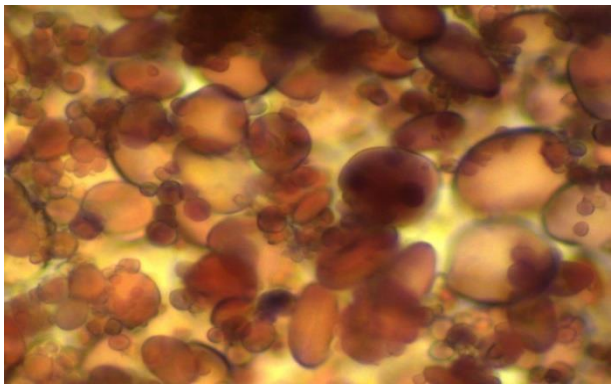


Figure 4. Microscope photo of emmer starch granules

It can be summarized that the alkaline method used was suitable for the isolation of starch from emmer, as an alternative raw material to the already known starch production. However, the results showed that alkaline treatment alone was not sufficient to produce starch with low protein content, which required enzymatic treatment. Thus, the selected levels of the amount of multienzyme complex and processing time showed well the rate of change of the amount of protein in the composition of alkaline isolated starch. By increasing the amount and time of enzymatic treatment, more satisfactory results were obtained, which in turn did not lead to changes in the structure of the starch granules.

Conclusions

A modification of a technology for the isolation of emmer starch from whole grains is proposed based on an alkaline technology for the production of rice starch in combination with enzymatic treatment. A multi-enzyme complex was used to further purify the starch due to its high protein content. A temperature and pH regime were established for optimal enzyme activity (temperature 65℃ and pH 7.9, in this particular case the temperature was chosen to be 55℃), after which the samples were

treated with different amounts of enzyme (from 0.05% to 2.2%) and different time (1-8 h). After the analyses carried out, it is clear that the alkaline method for a time of 48 h of alkaline soaking can be replaced by a combined time of 24 h and a minimum amount of enzyme and thus intensified. The results showed that increasing the enzyme treatment time and enzyme concentration resulted in a decrease in the protein content of the samples, with the percentage changes in the initial stages being greatest.

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