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ORIGINAL PAPER

Effect of added enzymes and quinoa flour on dough characteristics and sensory quality of a gluten-free bakery product

Annalisa Romano[1,2] · Paolo Masi[1,2] · Annachiara Bracciale[1] · Alessandra Aiello[1] · Maria Adalgisa Nicolai[1] · Pasquale Ferranti[1,3]

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Abstract
The study is aimed at developing a new cereal-based product, with increased nutritional quality, using quinoa flour. The effect of the use of transglutaminase (TGase) and proteolytic enzymes on the microstructure, properties and in vitro digestion of gluten-free bakery products based on quinoa flour was evaluated. Microstructural results evaluated by means of Scanning Electron Microscopy showed that the quinoa starch granules are rather small (0.4–2 µm) and the presence of TGase induced significantly changes in dough and baked samples microstructures. The overall acceptability of the breads was improved by TGase addition. The results achieved showed that these enzymes have different effects on the bread characteristics and may improve properties of formulations, setting the basis for the development of baked quinoa products.

Keywords *Chenopodium quinoa* Willd. · Quinoa proteins · Transglutaminase · Microstructure · In vitro digestibility · MS/MS analysis

Introduction

Celiac disease (CD) is an immune-mediated enteropathy triggered by the ingestion of certain cereals, including wheat, rye, barley, triticale and oats in genetically susceptible persons [1]. To develop gluten-free (GF) breads for celiac patients, a number of alternative flour types, such as corn, rice, cassava, soybean, chickpea, teff and pseudocereals (e.g. quinoa, buckwheat and amaranth) have been evaluated to substitute wheat flour [2–5]. Baking of GF flours is a challenge due to the lack of gluten proteins, as gluten is a protein which possesses structure-forming ability that affects elastic properties of dough and contributes to the overall appearance and crumb structure of many baked products. Therefore, the removal of gluten in GF formulation is a very demanding task resulting in often low quality, poor mouthfeel and low flavour products [6]. In recent times, there has been a growing interest in the use of quinoa flour as ingredient in bread formulation by replacing flour [7–11]. Pseudocereal quinoa (*Chenopodium quinoa* Willd.) is a native plant in the Andean region. Because of quinoa plants' stress-tolerant characteristics (cold, salt and drought tolerant) and its high nutritional value [12, 13] and biological properties quinoa has described as one of the grains of the twenty-first century and FAO launched the International Year of Quinoa in 2013. Quinoa cultivation has crossed continental boundaries to reach Europe. It is cultivated in France, England, Sweden, Spain, Denmark, Finland, Holland and Italy [14, 15]. It is grown in the United States and in Canada, as well as in Kenya, in the Himalayas and India [14]. Quinoa grains are considered as potentially gluten-free with an excellent nutrient profile [16, 17]. They contain considerable amounts of fibre and minerals, such as calcium and iron [16, 18]. Thus, the use of quinoa flours to develop gluten-free products and to improve their quality provides a promising step towards ensuring that celiac patients consume nutritionally balanced products.

While the nutritional value and the chemical composition of quinoa were characterized, several aspects concerning the technological applications have received less attention [10–12]. Overall, the baking quality is considered rather

✉ Annalisa Romano
annalisa.romano@unina.it

1 Department of Agricultural Sciences, University of Naples Federico II, Via Università 100, Portici, 80055 Naples, Italy

2 Centre for Food Innovation and Development in the Food Industry, University of Naples Federico II, Via Università 100, Portici, 80055 Naples, Italy

3 ISA, Avellino, Italy

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low due to the absence of gluten [12], flavour, texture and appearance of baked goods, including quinoa in the recipes were reported only as moderately acceptable [12].

The use of enzymes in bread making industry has the potential to improve the physicochemical, thermal and rheological properties of doughs and breads [19] of wheat and non-wheat flours. Enzymes are naturally present or deliberately added in foods as processing aids. There exists a wide range of enzymes and their blend depending on their effect and application [20, 21]. Enzymes are used as technological aids in different stages of baking because they are effective in reduced firmness of crumb, delay staling of baking, improve dough-handling properties and enhance bread quality [22]. In addition, the enzymes are a better alternative than chemical additives because they are regarded as safe.

Transglutaminase (TGase), a protein cross-linking enzyme, has been proposed as processing aid for modifying functional properties of many protein-based foods. In particular, TG has been reported to improve dough-handling properties and to increase fermentation stability, and loaf volume [23–25], because it catalyzes the formation of intra or intermolecular ε-(g-glutamyl) lysine isopeptide bonds [24]. Moreover, some authors [26, 27] describe the effect of enzymatic protein crosslinking on protein digestibility. In fact, it has been assumed that enzymatic modification of proteins leads to firmer matrices that are digested to a lower extent. Moreover, some authors [28] reported that a bacterial protease could improve the quality of gluten-free brown rice bread, in which the loaf volume increases and crumb hardness and chewiness decreases.

To our knowledge, scanty information are available in respect to use of enzymes in quinoa bread making for improving the baking quality of products. The most of them reported the use of different enzymatic treatments to increase the nutritional profile (e.g. the mineral availability) of quinoa flour, as phytase [29].

The objective of the present paper was to study the effect of three different enzymes (transglutaminase and two proteases) on the microstructure, nutritional and sensorial properties of a quinoa bakery product. Moreover, the in vitro digestibility of quinoa proteins was evaluated.

Materials and methods

Materials

White quinoa seeds were purchased in a local market. ACTIVA® EB Transglutaminase was purchased from Ajinomoto (Japan). Proteolytic enzymes, commercially referred to as Amano N and Europa 2, were purchased from by Amano Enzyme Inc. and Europe Enzyme Bioproduct, respectively. Amano N is an enzyme preparation from *B. subtilis* (enzyme activity > 150,000 U/g). Europa 2 is an enzyme preparation from *B. stearothermophilus*, enzymatic activity > 10,000 U/g.

Quinoa flour preparation

Quinoa seeds were desaponified carrying out a first soaking step in water at room temperature for 30 min and a second soaking for 20 min at 70 °C under continuous stirring. Desaponified seeds were dried in an oven at 60 °C for 4 h. Milling of dried seeds was performed using a variable speed laboratory blender (LB20ES, Waring Commercial, Torrington, Connecticut, USA), so that the flour would pass through a 425 µm stainless steel sieve (Octagon Digital Endecotts Limited, Lombard Road, London, UK). The flour samples were collected and stored in polyethylene bags at 4 °C until used for analysis.

Nutritional characteristics (%) of quinoa flour according to AACC methods 2000 [30] were 8.1 ± 0.1% moisture (gravimetric Method 44-19) and 13. 72 ± 0.2% proteins (Kjeldahl Method 46-30) ($N \times 5.96$).

Bread making process

The amount of all ingredients used (quinoa flour, water, salt, vanillin, yeast, sugar) in the four formulations was the same; the only difference in the formulation of the bakery products was been the type and the amount of enzyme. All doughs were prepared in a Brabender farinograph (O. H. Duisburg, Germany) using a 50-g bowl. The control dough was prepared by weighting 50 g of quinoa flour by means of a analytical balance (Sartorius BL 1500, Germany) and by adding them the deionised water (56%), yeast (3%), salt (2%), sugar (1%), vanillin (0.05%). Europa 2 and Amano N were added to control doughs at 0.50% (w/w), while Tgase concentration investigated was 1.5% (w/w).

Mixing time and temperature were kept constant and equal to 10 min and 25 °C, respectively. The dough was incubated at 36 ± 4 °C, 70% U.R. for 45 min of fermentation. Baking took place inside a conventional electric oven (Moretti Forni S.p.A., Pesaro, Italy) where temperature was kept under control at 190 °C for 40 min.

Microstructural analysis: Scanning Electron Microscopy (SEM)

Quinoa flour, doughs and bakery products were dried at the critical point and coated with gold particles in an automated critical point drier (model SCD 050, Leica Vienna). Microstructure of samples was examined by means of Scanning Electron Microscopy (LEO EVO 40, Zeiss, Germany) with a 20 kV acceleration voltage and a magnification of 5000× for flour samples and of 2000× for doughs and bakery products.

In vitro digestion model and ELISA gluten assay

For in vitro simulation of protein digestion process, samples were submitted to simulated in vitro digestion as previously reported [31] with minor modifications. Briefly, 1 mg of sample was dissolved in 5% formic acid at the concentration of 1 mg/mL and incubated at 37 °C with pepsin (1:100 enzyme/protein ratio, w/w) for 60 min. Before pancreatic digestion, the samples were evaporated and washed twice with deionised water. Trypsin (1:100, w/w), chymotrypsin (1:100, w/w), elastase (1:500, w/w) and carboxypeptidase (1:100, w/w) were added in 0.1 M sodium phosphate buffer (pH 7.0) and the mixtures incubated 1 h at 37 °C. The reaction was stopped by heating 5 min in a boiling water bath.

Gluten content of quinoa samples was determined using the R5 assay kit (R7001 Ridascreen Gliadin), according to manufacturer's instructions. A standard curve was built with gliadin at various dilutions (0–120 ng/mL). Quinoa samples were subjected to an extraction step with the 'cocktail solution' as suggested by the kit provider. Afterward, aliquots were diluted (1:50–1:200) in the dilution buffer (room temperature) and assayed in triplicate. Statistical analyses were carried out with the Excel 2010 software (Microsoft Co., WA, USA).

Nano LC–ESI–MS/MS analysis

The peptide solution was analysed by nano LC–ESI–MS/MS using a Orbitrap XL instrument (Thermo Fisher) equipped by a nano-ESI source coupled with a nano-ACQUITY capillary UPLC (Waters): peptide separation was performed on a capillary C18 column (0.075 mm × 100 mm; m, Waters) using aqueous 0.1% formic acid (A) and ACN containing 0.1% formic acid (B) as mobile phases. Peptides were eluted by means of a linear gradient from 5 to 50% of B in 45 min and a 300 nL/min flow rate. Mass spectra were acquired over *m/z* range from 400 to 1800; the ten most intense doubly, triply or quadruply charged ions detected in each spectrum underwent CID fragmentation (dependent scan acquisition mode) and MS/MS spectra were acquired over a *m/z* range from 50 to 2000.

Sensory analysis of bakery products (quantitative descriptive analysis)

The sensory profile of the four bakery products was determined according to Quantitative Descriptive sensory Analysis (QDA) approach proposed by Stone and Sidel [32].

Attribute terms for sensory evaluation of bakery products were developed by a panel of 12 semi-trained panelists, all of them were selected among the staff and graduate students of Department of Agricultural Sciences—Division of Food Science and Technology, between the age of 20–60 years, nonsmokers and with previous training in the use of descriptive terms [33], so they were experienced in QDA profiling. The judges generated sensory terms individually during three orientation sessions. Each attribute term was extensively described and explained to avoid any doubt about the relevant meaning. Finally, twelve attributes were selected by consensus (the frequency of citation > 60%) to describe the bakery products. After the terminology development phase, ten panelists were submitted to training for the evaluation of appearance, flavour and texture of bakery products and to familiarize themselves with scales and procedures. Appearance, colour of crust and crumb, air bubble structure, aromatic intensity, salty, sweetness, acidity, bitterness, vegetal aftertaste, cohesiveness and overall acceptability were considered as sensory attributes using a ten-point hedonic scale, anchored at the left and right extremes with the terms "0.0 = low/weak" and "9.0 = high/strong", respectively. The definition of each descriptor and the scale anchors are reported in Table 1. Before the sensory evaluation, samples were maintained at room temperature for 90 min after baking; samples were cut into 2.0 cm thick slices and divided in four smaller pieces. Samples were coded, presented with random three-digit codes in identical containers, and the four samples were analysed in the same section. Each sample was served with warm water to rinse mouth between evaluations of the bakery products.The final scores of each attribute are presented as the mean value of the results from the ten panelists.

Statistical analyses

All experiments were performed in triplicate samples. Statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance was conducted to evaluate the effect of variety on parameters during cooking processes. Significant differences between the detected parameters were compared by means of Duncan's multiple comparison test at the 95% confidence level ($p \leq 0.05$). In addition, a principal component analysis (PCA) was carried out to visualize possible relationships within the data matrix. QDA datasets were arranged in a matrix of *i* lines (samples) and *j* columns (attributes), and principal component analysis (PCA) was carried out as reported by Alencar, Carvalho de Morais, Steel and Bolini [34].

Results and discussion

Microstructural characteristics of quinoa flour

A representative scanning electron microscopy (SEM) image of quinoa flour is shown in Fig. 1. Morphological features of quinoa flour showed starch granules, varying

Table 1 Descriptive terms, definitions and anchors used in the sensory analysis of bakery products

Descriptors	Definition	Anchor	
		Min (0)	Max (9)
Appearance	Overall appearance of the product	Poor	Excellent
Crust colour	Colour tone and intensity of crust	Same colour of crumb	Dark brown
Crumb colour	Colour tone and intensity of crumb	Pale brown	Dark brown
Air bubble structure	Presence and amount of air bubbles in the product	Low	High
Aromatic intensity	Global taste intensity of the crumb	Weak	Strong
Salty	Basic taste associated with sucrose	Weak	Strong
Sweetness	Basic taste associated with sucrose	Weak	Strong
Acidity	Basic taste associated with citric acid	Weak	Strong
Bitterness	Taste related to the presence of bitter compounds	Weak	Strong
Vegetal aftertaste	The intensity of vegetal plant flavour	Low	High
Cohesiveness	The way the crumb reacts when broken by fingers	Poorly cohesive it crumbles	Very cohesive it sticks
Overall acceptability	The overall impression of the product based on all the sensorial attributes tested	Low	High

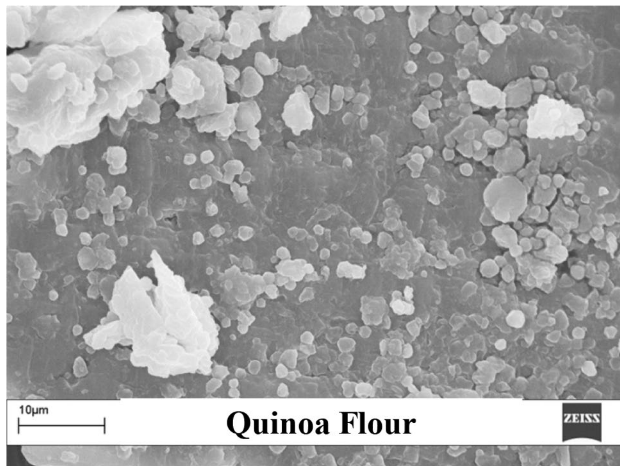

Fig. 1 Scanning electron micrographs (SEM) of quinoa flour

in shape from polygonal, angular to irregular (Fig. 1). The size of quinoa starch granules was mostly in the range of 0.4–2.0 µm (Fig. 1), as reported also by other authors [18, 35, 36]. The size of starch granules is usually larger than that of lipid and protein bodies [27, 37, 38, 39], thus the larger globular structures found in Fig. 1 can be identified such as starch granules. Quinoa starch may present as aggregations (Fig. 1). These spherical- or oblong-shaped aggregates were between 10 and 30 µm in size (Fig. 1), in agreement with data of [18, 40, 41, 42]. The formation of these aggregates may be largely due to the presence of protein because adding pepsin facilitated their disaggregation [35, 41].

Effect of added enzymes on microstructural characteristics of products after leavening and baking processes

To study the effects of enzymes on microstructural properties of samples made with Europa2, Amano N and TGase, the characterisation of the sample microstructure after leavening and baking processes by means of SEM was performed.

Figure 2 shows representative SEM micrographs of control dough and doughs with enzymes. As expected starch granules were less well visible and discernible in microstructure of doughs (Fig. 2) than initial quinoa flour (Fig. 1). The reference dough in fact exhibits a pronounced protein matrix with embedded starch granules. During mixing, proteins start to interact with each other through hydrogen, ionic, hydrophobic and covalent bonds which lead to the formation of a cross linked network [43]. The addition of Europa2 and Amano N enzymes in the dough formulation (Fig. 2b, c) did not change dramatically the protein matrix of control dough (Fig. 2a). Moreover, the presence of TGase caused significant changes in dough microstructure. The microstructure of dough with TGase (Fig. 2d) presented, in fact, a more developed gluten network structure, as compared to the other doughs (Fig. 2a–c), which might be attributed to the formation of TG-catalysed heteropolymers. A more organized structure was found by Renzetti et al. [44] that studied the transglutaminase impact as a sole treatment on baking performance of buckwheat and brown rice, by [45] that studied the glucose oxidase effect on wheat flour dough at molecular level and also by [27] that studied the impact of transglutaminase on microstructure of white beans (*Phaseolus vulgaris* L.).

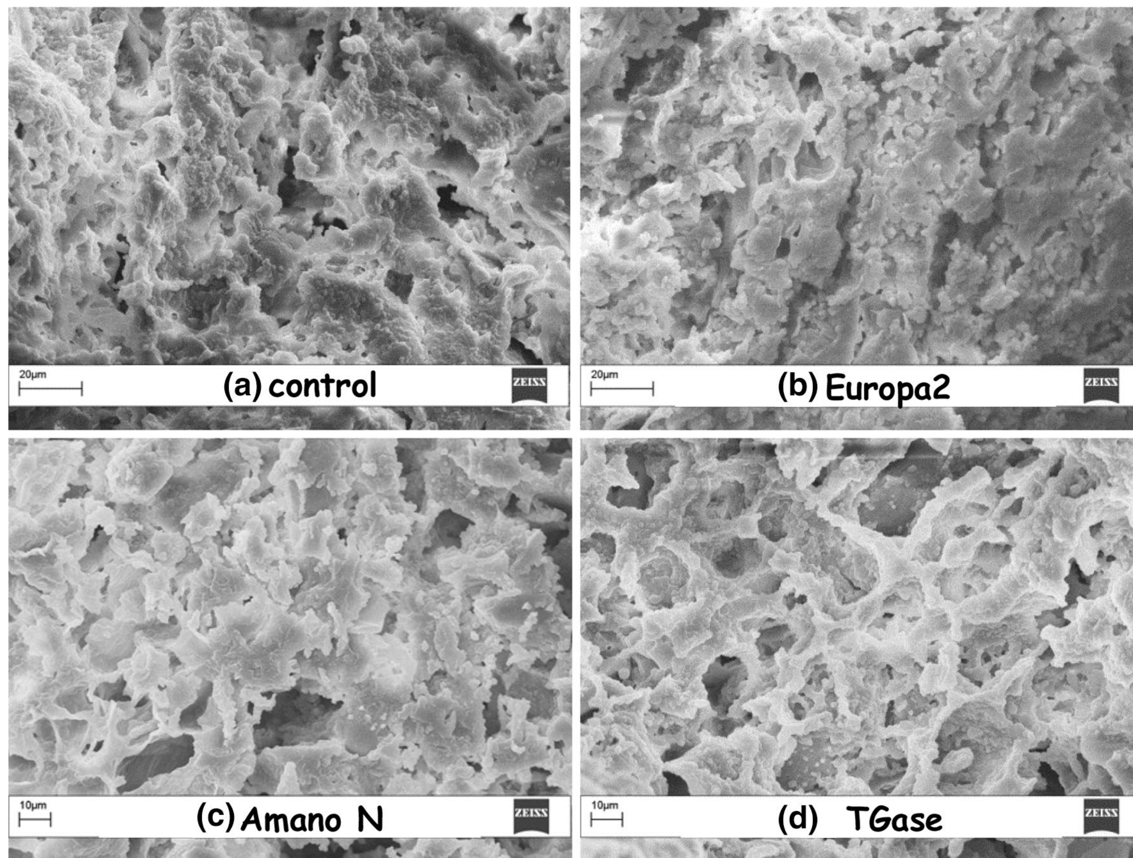

Fig. 2 Scanning electron micrographs (SEM) of doughs after leavening: **a** control; **b** with Europa 2; **c** with Amano N; **d** with TGase

SEM images of baked samples are shown in Fig. 3. All samples showed small starch granules coated by a continuous protein matrix. The gas cells (black arrows, Fig. 3) were visible, mostly in Europa 2 and TGase samples. The control and Amano N products (Fig. 3a, c) showed in fact denser microstructure than Europa 2 and TGase ones (Fig. 3b, d). In particular, the addition of TGase caused the formation of cavities and micropores through the protein phase of sample (Fig. 3d), which would indicate the protein polymerization described previously in dough microstructural results.

Protein electrophoretic analysis

In Fig. 4, the electrophoretic profiles of proteins extracted from quinoa flour and baked samples, (control and products with added enzymes), were reported. The most abundant polypeptide components presented a molecular mass in the range 30–40 and 20–25 kDa (Fig. 4), which corresponded, respectively, to the acid and basic subunits of 11S globulins. By comparison with other studies [46], the polypeptides of molecular weight of about 15 kDa corresponded to 2S albumins. In quinoa protein isolate, profile the intensity of the bands increased and that confirmed its high grade of purity. The protein fraction soluble at pH 5 contained 11S globulin chains, but especially 2S albumins. Data were confirmed by HPLC analysis (not shown).

In vitro gastrointestinal digestion and d gluten assay

LC-MS/MS analysis of GI digests (Fig. 5) showed that only a few peptides survived simulated digestion process indicating the high digestibility of quinoa proteins. Protein database search of peptide sequences did not allow to assign the parent proteins, as quinoa protein sequences are nearly absent in protein databases, with the exception of legumin 11S [47]. ELISA assay R5 excluded the presence of gluten in quinoa isolate and in quinoa flour at level higher than 3 ppm.

Sensory profile of the bakery products

To evaluate the potential effect of added enzymes on sensorial profile of a gluten-free bakery product, Quantitative Descriptive Analysis (QDA) was used to evaluate the sensory aspects of the gluten-free bakery products. Figure 6 shows the mean scores of the 12 sensory attributes

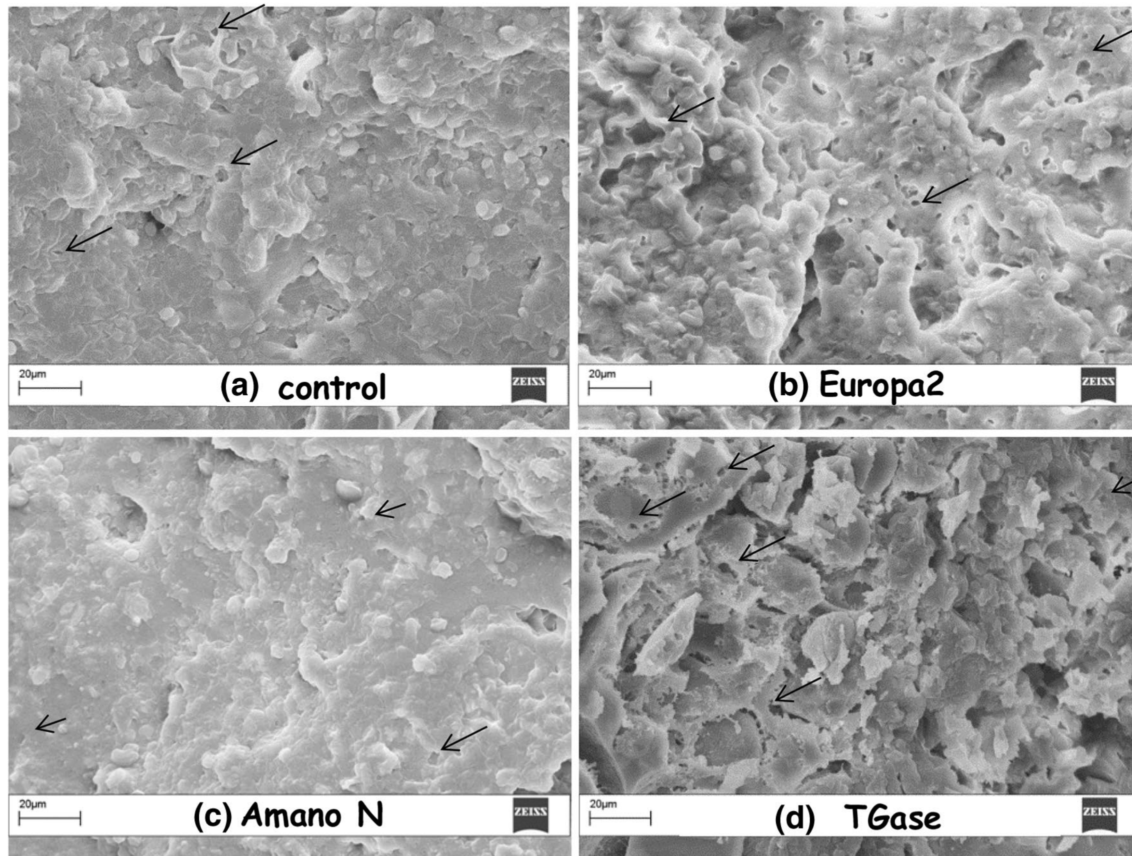

Fig. 3 Scanning electron micrographs (SEM) of bakery products: **a** control; **b** with Europa 2; **c** with Amano N; **d** with TGase

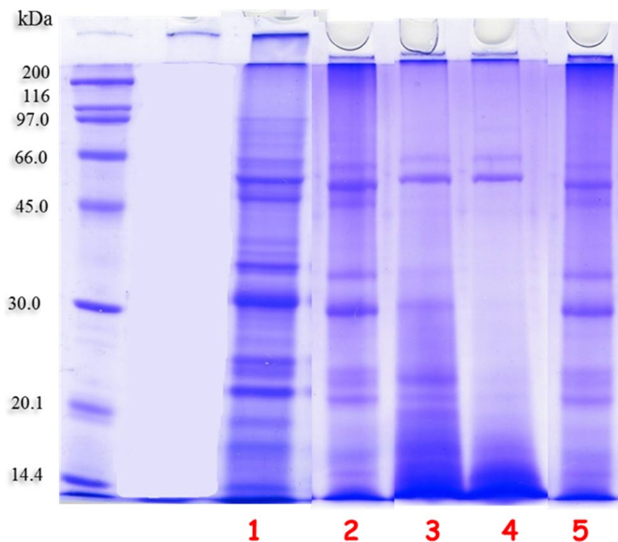

Fig. 4 SDS-PAGE analysis of proteins from (1) quinoa flour and from quinoa bakery products: (2) control; (3) with Europa 2; (4) with Amano N; (5) with TGase

(appearance, colour of crust and crumb, air bubble structure, aromatic intensity, salty, sweetness, acidity, bitterness, vegetal aftertaste, cohesiveness and overall acceptability) selected by QDA to describe the samples.

With reference to samples aspect, significant appearance differences could be observed, in fact it was possible to discriminate ($p < 0.05$) TGase (7.1 mean intensity ratings) and Amano N (5.2 mean intensity rating) samples from control and Europa 2 samples.

In terms of colour differences, no significant differences ($p < 0.05$) were observed between control and TGase samples (Fig. 6), while a significant increase in crumb and crust colour ($p < 0.05$) of samples resulted from addition of the Europa 2 and Amano N enzymes. The presence of Europa 2 and Amano N enzymes contributed significantly to darkening crust and crumb colours of the bakery products. Browning can be associated with greater occurrence of the Maillard reaction into doughs with Europa 2 and Amano N during baking due to a higher number of free amino groups upon protease action.

The samples produced with quinoa flour presented the lowest scores for the air bubble structure attribute and differed statistically ($p < 0.05$) from the samples with added

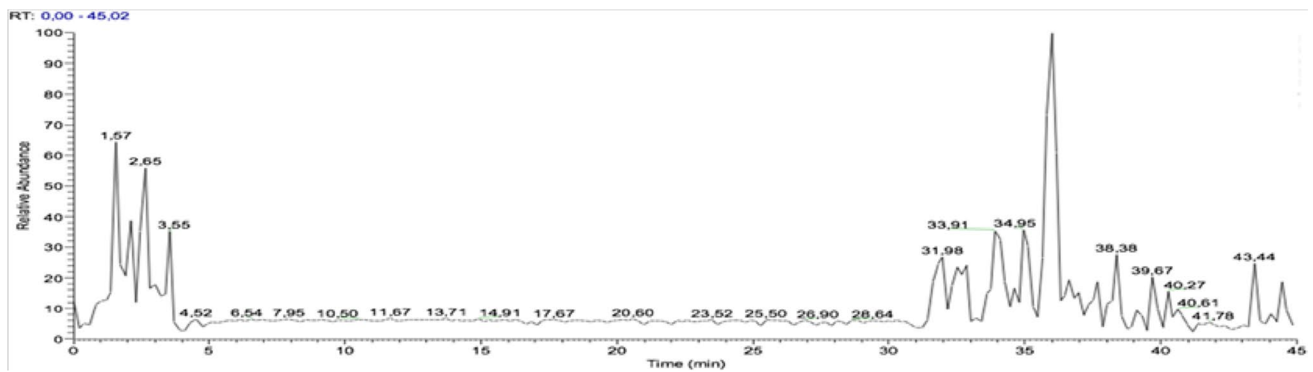

Fig. 5 LC ESI Q-ToF Orbitrap MSMS chromatogram of quinoa protein isolate gastrointestinal digest

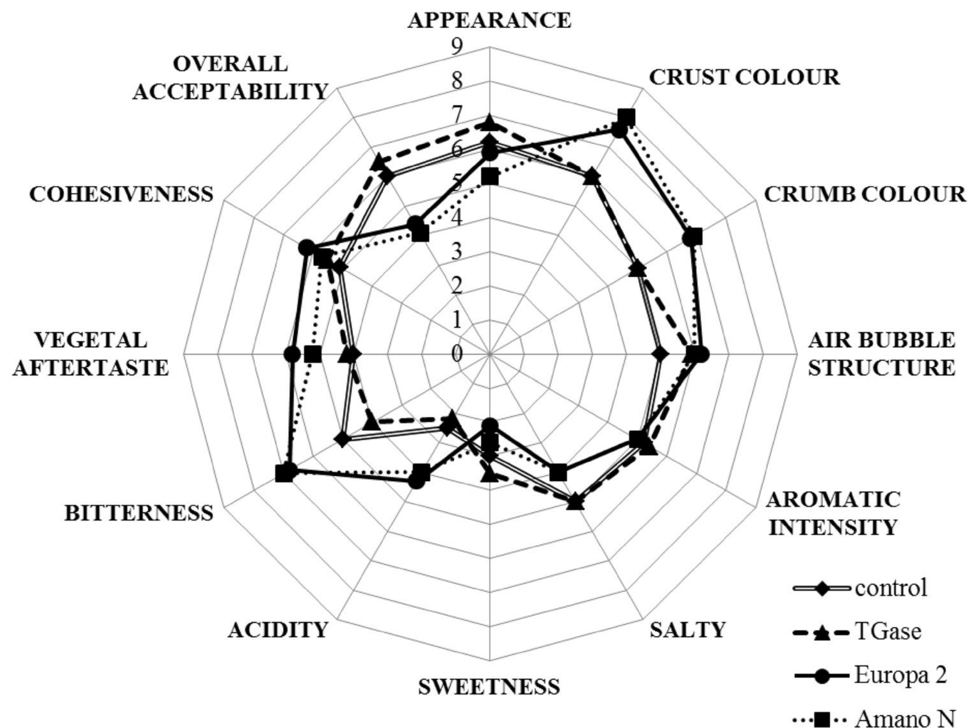

Fig. 6 Spider diagrams representation of QDA analysis of bakery products: filled diamond: control; filled triangle: with TGase; filled circle: with Europa 2; filled square: with Amano N

enzymes. Indeed, air bubble structure of samples with added enzymes was finer and more uniform than control.

When focusing on odour, aromatic intensity remained similar to that of the control for all samples. In terms of taste attributes, Europa 2 and Amano N samples were characterized by a lower salty and sweetness intensity (5 and 4 mean intensity ratings, respectively) than control and TGase samples. Detectable levels of acidity (< 4.3 ratings) were perceived in all samples.

In relation to bitterness, the gluten-free breads containing Europa 2 and Amano N presented the highest scores and mean intensity rating > 6 (Fig. 6), likely due to the production of bitter peptides from quinoa proteins by action of food grade proteases.

Similarly, significant differences ($p < 0.05$) were observed for vegetal aftertaste descriptor exhibiting in Europa 2 and Amano N samples evaluation (> 5.2 ratings) from control and TGase samples (< 4.4 ratings).

In the case of cohesiveness, samples with Europa 2 and Amano N enzymes were more cohesive (ranged from 5.8 to 6.2) than TGase (5.5) and control (5.2), suggesting a more integrated matrix, less susceptible to crumble and fracture [48]. Low cohesiveness negatively influences consumer's acceptance of bakery products, because it results in high crumbling. From this point, the enzymes addition could decrease the crumbling, therefore, improve the sensory quality. Besides high cohesiveness values are desirable for industry scale producers and bread distributors [21].

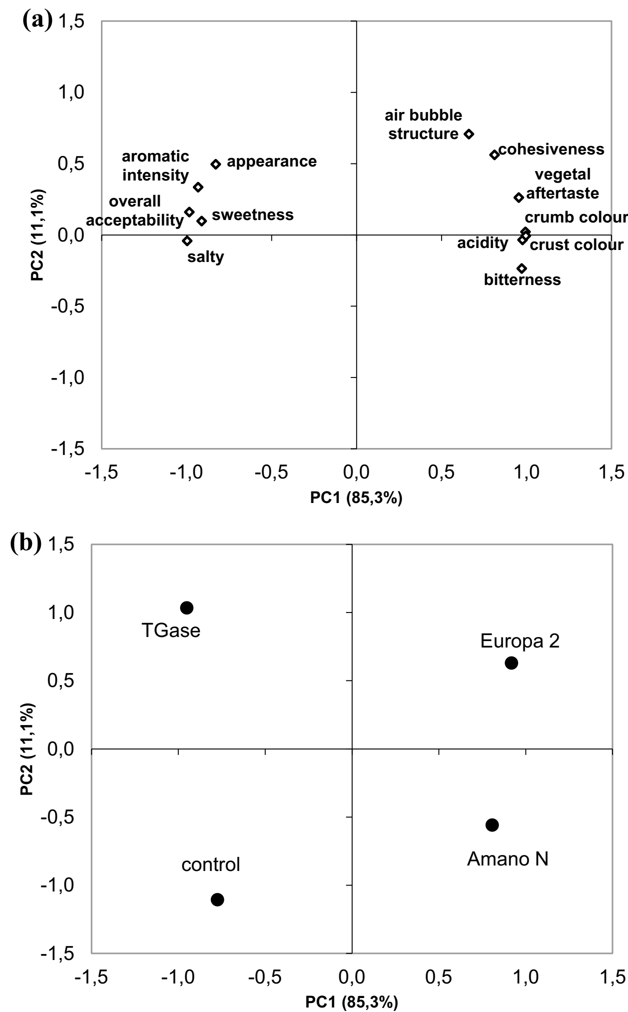

Fig. 7 Results of PCA loading and score biplot for bakery products (control, with TGase, with Europa 2, with Amano N)

In terms of overall acceptability, samples with TGase and control were more acceptable to the panel than samples with Europa 2 and Amano N, which were not significantly different ($p < 0.05$) between them as presented in Fig. 6. Actually, bread produced with pseudocereals (quinoa, amaranth and buckwheat) are moderately accepted by consumers, as reported by [34, 49].

Additionally, the QDA data were analysed by Principle Component Analysis (PCA) to give an overview on sensory profile of bakery products. Figure 7 showed plots of loadings (Fig. 7a) and scores (Fig. 7b) obtained from PCs, where the first two principal components (PC1 and PC2) accounted for 96% of the total variance of the data. In particular, the principal component PC1 explained 85% of the variation of the data, while the principal component PC2 explained 11%. The score distribution from the two first PCs allowed for clustering of the samples into four groups (Fig. 7b). In particular, were positively scored on PC1 and associated with sensory parameters, such as air bubble structure, cohesiveness, vegetal after taste, colour of crumb and crust, acidity and bitterness. In the positive PC1 of the loadings plot (Fig. 7a), cohesiveness, vegetal after taste, colour of crumb and crust, acidity, bitterness (correlation values higher than 0.8) and air bubble structure (eigenvalue of 0.7) were the sensory parameters with highest scores for samples with proteolytic enzymes (Europa 2 and Amano N). Moreover, TGase samples, showing negative scores on the PC1 and entirely located in the positive part of PC2, were characterized by appearance, aromatic and sweetness intensity and overall acceptability (Fig. 7a). The last group, having negative scores on both PC1 and PC2, was represented by control (Fig. 7b).

According to the results presented, however, more studies are needed with added enzymes in bakery products to obtain samples with higher sensory acceptance for this type of product.

Conclusions

Bread, exclusively based on quinoa flour with valid nutritional and sensorial properties, was obtained. These characteristics can be optimized with the addition of processing enzymes, such as TGase or proteolytic enzymes. Use of food grade proteases improved the characteristics of dough, but induced production of bitter peptides and darking colour (crust and crumb) of samples. The incorporation of TGase improved sample appearance and taste. In conclusion, sensory attributes selected by QDA showed the best results for TGase bakery product.

The achieved results confirm the interest in studying the use of enzymes in the production of gluten-free bread based on quinoa flour. However, a more detailed knowledge on the use of enzymes in the production of gluten-free bread based on quinoa flour and on the allergenic properties of the product obtained needs to be acquired.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

Compliance with ethics requirements All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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