FACULTY OF BIOSCIENCE ENGINEERING

Tef [*Eragrostis tef* (Zucc.)Trotter] protein characterization and its suitability as a gluten-free cereal

Number of words: 20 623

Sieglinde Pattyn
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Promotor: Prof. dr. ir. Katleen Raes
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Master's Dissertation submitted for obtaining the degree of Master of Science in Biochemical Engineering Technology

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Kortrijk, June 2017

Author,

Sieglinde Pattyn

Promotor,

Katleen Raes

Tutor,

Habtu Shumoy Abraha

Foreword

Before starting to read this thesis, I would like to have your attention for a little preface.

I am a master student of industrial engineering in the biochemistry. For my thesis I did a research entitled as “Tef [*Eragrostis tef* (Zucc.)Trotter] protein characterization and its suitability as a gluten-free cereal”. During a period of four months I have worked on the practical aspect of this research. By the end of June 2017 the writing part of this thesis was finished.

First of all, I would like to thank my promotor Katleen Raes for her excellent guidance during the whole process. Next, I thank my tutor Hubtu Shumoy for his support from the beginning to the end of the work. It was a pleasure to perform this research together with him. Also it was a honor to work together with all internship and non-internship students in the laboratory and therefor I would like to thank all for the wonderful experience they gave me. The whole process was very interesting and is something to cherish for the rest of my life.

To end, I would like to mention my family and friends for their unlimited support, not only during my thesis research but also during the whole period of my educational journey. Without them, I would never have gotten so far.

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Abstract

In this research, a protein characterization was performed on seven different varieties of *Eragrostis* tef. First the crude protein content varied from 8.48-9.40 g/100g of flour on dry basis (p-value < 0.001). SDS-PAGE showed no difference in protein pattern between the different tef varieties. Next, the content of the Osborn fractions decreased in the order of globulin (9.55-12.93%) > albumin (5.43-8.68%) > prolamin (1.98-2.49%) > glutelin (0.25-0.59%). SDS-PAGE showed a clear difference in protein pattern between the different Osborn fractions indicating a good Osborn extraction. As the recovery was very low, further optimization of the Osborn fractionation for tef might be necessary. In vitro protein digestibility (IVPD) was tested on flour, with results ranging from 70.9-72.2%. IVPD of *injera* was found to be a little bit higher (72.2-74.32%). SDS-PAGE showed that the main difference in protein pattern between tef flour, fermented dough and *injera* was notable after the fermentation step. No difference was found between the fermented dough and *injera.* By the use of a commercial enzyme linked immunosorbent assay (ELISA) kit the issue that tef is a gluten-free cereal was confirmed. For all seven tef varieties, the gluten content was found to be below 20 mg/kg. Further research on the presence of the peptides in tef prolamin to which the monoclonal antibody reacts is necessary. Other possible allergens based on the SDS-PAGE results, divided according to plant food allergens, are allergens from the cupin superfamily, the prolamin superfamily, plant defense system, and enzymes. SDS-PAGE performed on fermented dough and *injera* showed a strong decrease in possible allergens. For the future, based on this protein characterization, tef might replace wheat in food products. Although, further research on other compounds (eg. carbohydrates, lipids, fibres…) and baking performances of tef are necessary.

Abbreviations

Ama r	*Amaranthus retroflexus*
Ama v	*Amaranthus viridis*
Ave s	*Avena sativa*
BDAI	Barley dimeric alpha-amylase inhibitor
BTI	Barley trypsin inhibitor
BMAI	Barley monomeric alpha-amylase inhibitor
CD	Celiac disease
DW	Dry weight
EFSA	European food safety authority
ELISA	Enzyme-linked immunosorbent assay
Fag e	*Fagopyrum esculentum*
FAVV	Federal agency for safety in the foodchain
FW	Fresh weight
HLA	Human leukocyte antigen
Hor v	*Hordeum volgare*
IgE	Immunoglobulin E
IUIS	International union of immunological societies
IVPD	In vitro protein digestibility
Lol p	*Lolium perenne*
LTP	Lipid transfer protein
MALDI-TOF-MS	Matrix assisted laser desorption/ionisation time-of-flight analyser mass spectrometry
Mr	Relative molecular mass
ND	Not determined
Pan mi	*Paniceae miliaceum*
LQPFP	Leucine-glutamine-proline-phenylalanine-proline
QLPFP	Glutamine-leucine-proline-phenylalanine-proline
QQPFP	Glutamine-glutamine-proline-phenylalanine-proline
QQQFP	Glutamine-glutamine-glutamine-phenylalanine-proline
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec c	*Secale cereale*
Set it	*Setaria italica*
Tri a	*Tricicum aestivum*
Tri m	*Triticum monococcum*
Tri p	*Trisetum paniceum*
Tri tp	*Triticum turgidum ssp. polonic*
Tri ur	*Triticum Urartu*
U	Unit
WHO	World health organization
Zea m	*Zea mays*

1 Introduction

Eragrostis tef is a cereal originated from Ethiopia (Wrigley et al., 2016). The seeds of the tef plant are among the smallest of cereals (Belay et al., 2009). According to Wrigley et al. (2016), the mass of one tef grain is only 0.6-0.8% of the total mass of a wheat grain. Because of this, tef grain is milled into whole-grain flour. This results in a much higher content of fibre and other nutrients such as minerals, vitamins and bioactive phenolic compounds than most other cereals (Gebremariam et al., 2014). The color of tef seed varies from white to dark brown. Tef in Ethiopia, is mainly used to make a traditional fermented-circular soft bread called *injera* or flat bread (Tatham et al., 1995). Other than Ethiopia, there was no interest in tef in the rest of the world for centuries. Now-a-days, there is a worldwide interest in cultivating this cereal. The Netherlands was for a long time, the only country cultivating tef in Europe (Wrigley et al., 2016). Currently, there is also a pilot experiment to cultivate it in Belgium. The high global demand of tef is a result of its high levels of essential amino acids, gluten-free nature and high mineral contents (Zhang et al., 2016).

All around the world, cereals provide an important part of the human nutrition. Cereals consists of five main components namely carbohydrates, proteins, fat, fibre and minerals (Wrigley et al., 2016). In this study, the properties of tef proteins were investigated. Plants provide about 40% of the total protein intake in adolescents (Lin et al., 2015). These proteins are mostly derived from cereals like corn, wheat, rice, sorghum and millet (Ustunol, 2015). Previous research on tef has led to very different results in protein characterization. In this thesis, research was performed on seven different tef varieties: Boset, Dega, Simada, Quncho, Tsedey, Zagurey and Zezew.

Next an in vitro protein digestibility (IVPD) was performed on tef flour and *injera*. No information about in vitro protein digestibility on tef was found in literature. This new information is now available in this research. By testing the IVPD on *injera*, the effect of food preparations (fermentation and baking) on IVPD was also tested.

Wheat and maize are the most dominant cereals grown all over the world. Nevertheless, problems like allergens, intolerances and celiac disease (CD) occur more and more in these cereals during the last decades (Spaenij-Dekking et al., 2005). Celiac disease is a lifetime gluten-sensitive autoimmune disease of the small intestine (Gujral et al., 2012). The only solution for these patients is a lifetime gluten free diet (Spaenij-Dekking et al., 2005). Gluten are proteins composed out of prolamins and glutelins (Singh et al., 2001). These two protein fractions are the most dominant ones in cereals like wheat and maize (Kumagai, 2010). The European Commission has set rules about food labelling. Products containing less than 20 mg/kg gluten can be labelled as gluten-free products. Several researches have already issued the suitability of tef for people intolerant/allergenic to gluten. Only one epidomological study was performed in The Netherlands. During this study, people responding on gluten are directly tested and examined after consumption of tef. This information was only an indication of tef to be gluten-free and is not completely reliable. Different from these previous researches, this thesis contains the amount of gluten in tef. According to these results and the rules set by the European Commission, tef can now be labelled correctly.

Beside gluten, also other allergens can occur in cereals. Plant food allergens are classified into the cupin superfamily, prolamin superfamily, plant defense system, structural proteins and storage proteins. In literature, no information about the presence of one of these allergens in tef was found. In this thesis, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in order to compare the molecular weights of the tef proteins with allergens in other cereals and pseudocereals found in literature. This also gives an indication of the possible presence of allergens in tef.

2 Literature review

2.1 Tef: the Ethiopian cereal

2.1.1 Classification

Cereals are an important part of the human diet all over the world. Cereals are part of the *Poaceae* (grasses) family which is divided into four subfamilies (Fig. 2.1): the *Ehrhartoideae* (rice), the *Chlorodoideae* (ragi and tef), the *Panicoideae* (maize, millet and sorghum) and *Pooideae* (wheat, barley, rye, oats) (Tatham & Shewry, 2008).

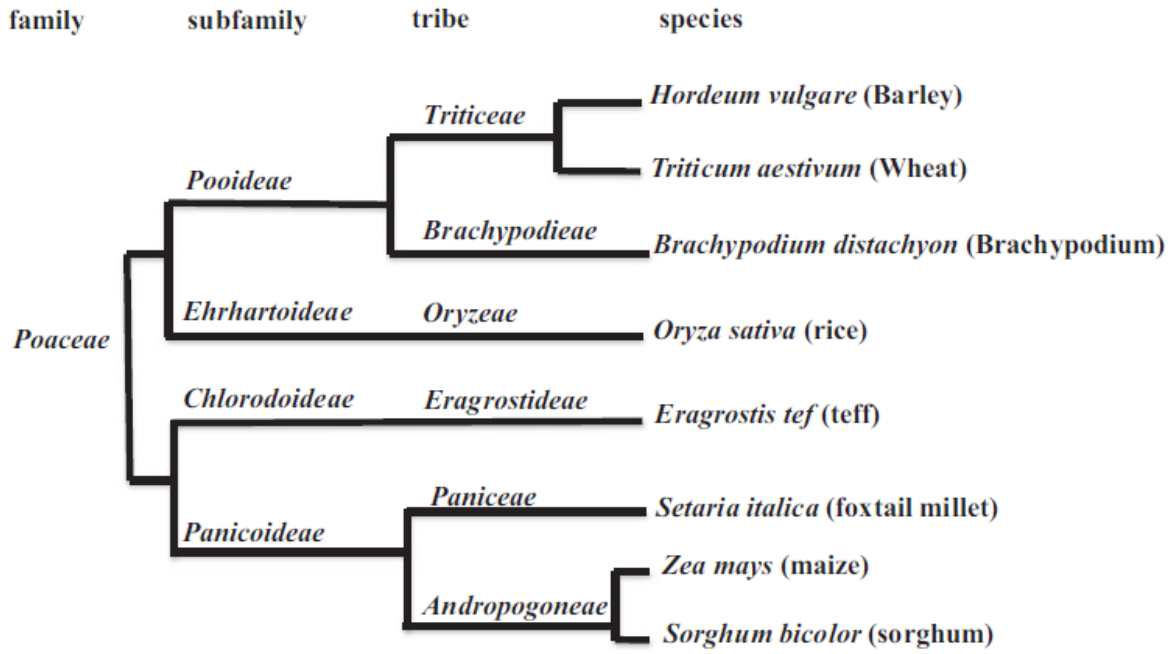


Figure 2.1: Phylogenetic tree of the *Poaceae* family (Zhang et al., 2016)

2.1.2 Origin and history

Eragrostis tef is known to be originated in Ethiopia. Ethiopia was the only country where tef is used for human consumption. Now-a-days, countries such as The Netherlands, some parts of the USA and South Africa are also using tef for human consumption. In Europe, the Netherlands was for a long time, the only country cultivating tef (Wrigley et al., 2016). Now, cultivation is also started in Belgium. According to Central Statistical Agency (2016), in Ethiopia tef covers about 23% of the total cultivated area. The history of tef goes back to the highlands of Ethiopia in the Neolithic times about 2600 BC. Until the 19th century, tef never left Ethiopia (Tatham et al., 1995). Only in the last decades, there has been a worldwide interest in tef. The reason for this is the belief of low toxic epitopes against celiac disease patients, high levels of essential amino acids (lysine and methionine) and high level of minerals (calcium and iron) (Zhang et al., 2016).

2.1.3 Physical properties of tef

There are different tef varieties with different colors varying from white to dark brown. For most varieties, the plant height is about 50-120 cm (Fig. 2.2). One tef plant is capable of producing about 9000-90 000 grains, depending on the variety and production conditions. The seeds are small and oval-shaped (length: 0.9-1.7 mm; diameter: 0.7-1.0 mm) (Wrigley et al., 2016). Three thousand tef grains have a mass of approximately one gram (Whole grains council, 2016). Compared to wheat, the mass of the tef grain is only 0.6-0.8% (Wrigley et al., 2016). The seeds of the tef plants are probably the smallest among cereals, with hundred kernels weighing from 0.18 until 0.38 mg (Belay et al., 2009).

Figure 2.2: Tef plant and grain (Berhe, 2009)

The optimal conditions to cultivate tef are 1800-2100 m above sea level, 10-27°C, an annual rainfall of 750-850 mm, and a rainfall of 450-550 mm during the growing season. The grain can be harvested when the vegetative and reproductive parts turn yellow or straw in color. Harvesting has to take place at the right time. Too late harvesting leads to a significant loss because of shattering and also the natural color of the grain can fade. Too early, on the other hand, makes the grains become moldy or they can sprout as tef is a very moisture sensitive cereal (Wrigley et al., 2016). Water is a stress factor occurring in a lot of plants. The effects are depending on the period of occurrence. Moisture stress at the beginning of the cultivation season affects the germination. At the end of the season, it affects the seed setting and seed quality. It not only affects the amount of grains, but also the size and weight of each individual grain (Mengistu & Mekonnen, 2012). On the other hand, an increase in moisture content can lead to the growth of fungi producing mycotoxins (Ayalew et al., 2006). The most important mycotoxins related with cereals are aflatoxins, ochratoxin A, fumonisins, deoxynivalenol and zearalenone. In overall food processing reduces the mycotoxin concentrations but no clompete elimination is achieved (Bullerman & Bianchini, 2007). Ayalew et al. (2006) found two main mycotoxins occurring in tef grains. The presence of aflatoxin B1 occurred for 22.9% of the tef grains (average content 5.1 µg/kg) while the incidence in wheat is 4.2% (average content 8.7 µg/kg). Another important mycotoxin is ochratoxin A, occurring in 27.3% of the tef grains with an average content of 32.7 µg/kg. Compared with wheat, the prevalence is 23.4% with an average content of 19.6 µg/kg. For this second mycotoxin, maximum levels of 5 µg/kg are set in 29 different countries. The high prevalence of both mycotoxins and the high daily consumption of tef are of great concern for human health in Ethiopia (Ayalew et al., 2006). Several steps of the cereal processing can influence the amount of mycotoxins. Sorting, trimming and cleaning reduces the amount of mycotoxins because of the removal of contaminated material but it does not destroy mycotoxins. In our case, as tef is milled into whole grain flour this is less relevant. During milling, no mycotoxins are destroyed but this preparation step redistributes the mycotoxins in the product. Effects of thermal processing is depending on the heating type and type of mycotoxin. Most mycotoxins are moderate stable in most food processing. Cooking, roasting, extrusion cooking at temperatures above

150°C lowers the amount of mycotoxins. Fumonisins are probably an exception, because for this mycotoxin the results were unclear (Bullerman & Bianchini, 2007). According to Ayalew et al. (2006), fermentation and baking have generally no effect on the present mycotoxins.

Tef can be cultivated under harsh environmental conditions where most other cereals cannot be cultivated. In Ethiopia, harvest is performed with a sickle and gathered in batches until it is ready for threshing. During the whole process there are a lot of losses, accounting for about 25-30%. The increased use of mechanical threshers decreases these losses significantly. The mechanized farming technologies used to harvest other cereals are not always achievable for tef because of its very thin and short plant stems and the small dimensions of the grain (Wrigley et al., 2016).

The anatomy of all cereals is almost the same (Fig. 2.3). Only a few specifications make the tef grains distinguish themselves from other cereals. The outside of a tef grain consists of a thin pericarp (Fig. 2.4). The pericarp is built out of a cuticle followed by a slime layer rich of pectins. The function of this layer is to absorb and to maintain moisture for later moisture stress. The pericarp is followed by a fused layer of mesocarp and endocarp containing some starch granules. Next, there is a seed coat called testa. In this layer, some tef varieties contain small amounts of tannins resulting in a thicker layer than normal. Next to the testa, there is a thick aleurone layer which is rich in proteins and contains lipid bodies. The germ or embryo occupies a large part of the grain and is rich in proteins and lipids. Finally, in the centre of the grain, there is also an endosperm consisting out of different layers. The outer layer contains most of the protein reserves and several starch granules. The thin-walled cells of the inner layer contain mostly starch granules and a few protein bodies. Compared to wheat, tef protein bodies are individual entities that do not coalesce to form a matrix (Wrigley et al., 2016).

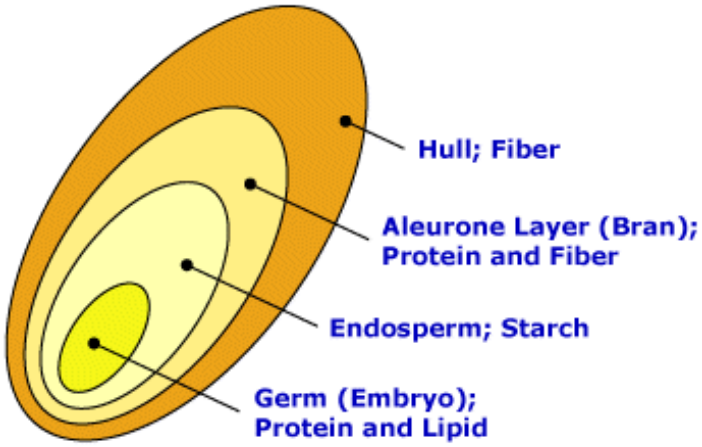


Figure 2.3: Anatomy of grains (Oregon State University, 2004)

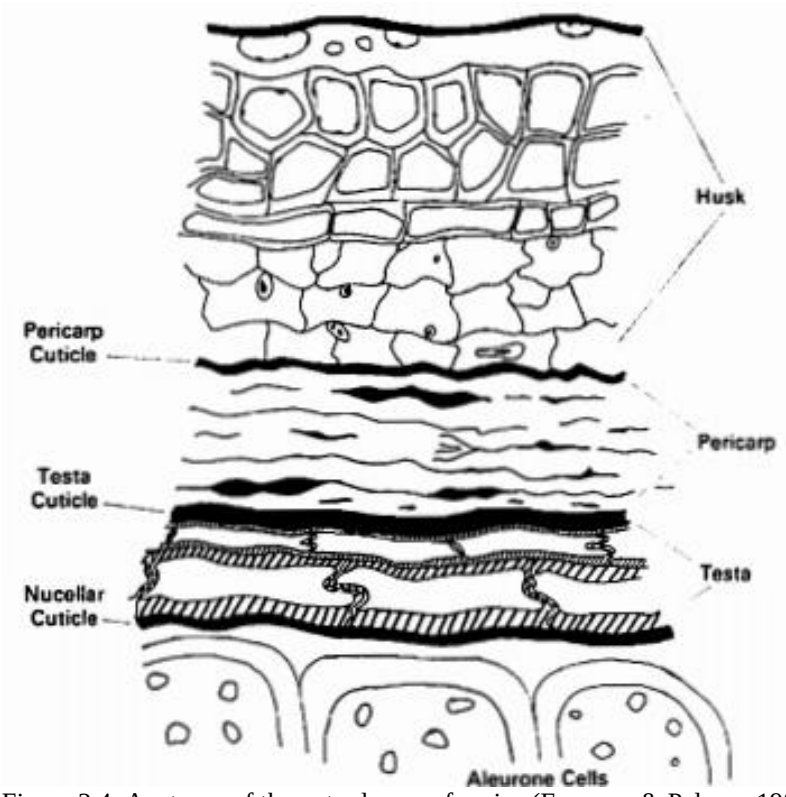


Figure 2.4: Anatomy of the outer layers of grains (Freeman & Palmer, 1984)

2.1.4 Use of tef as human food

Because of its small size, tef grains are milled into whole-grain flour (bran and germ included). This results in a much higher content of fibres and other nutrients such as minerals, vitamins and bioactive phenolic compounds than most other cereals (Gebremariam et al., 2014). Also, the nutritional value of tef is similar or even higher than that of wheat (Spaenij-Dekking et al., 2005). The energy value of tef is 1406 kJ per 100 g of flour (Wrigley et al., 2016).

Cereals are composed of five main components namely carbohydrates, proteins, fat, fibre and ash (Wrigley et al., 2016). The content of these five fractions can be found in table 2.1, according to three different researches. The results of these researches are quite comparable.

Table 2.1: Proximate composition of tef grain

Biochemical class	g/100g dry weight (Wrigley et al., 2016)	g/100g fresh weight (Gebremariam, 2014)	g/100g fresh weight (The National Academies, 1996)
Protein (N x 6.25)	9.4-13.3	8.7-11.1	9.6
Carbohydrate	73.0	73.0	73.0
Crude fibre	2.0-3.5	2.6-3.8	3.0
Fat	2.0-3.1	2.0-3.0	2.0
Ash	2.7-3.0	1.99-3.16	2.9
Moisture	/	9.30-11.22	11.0

Flours are never consumed by humans as a raw product (Tatham & Shewry, 2008). They are often exposed to fermentation or heat processes. During a fermentation process, microorganisms and their enzymes attend to biochemical modifications of the flour. This leads to changes in texture, taste, aroma, nutritional value and digestibility of the food products. In the industry it is also used as a preservation process because it extends the shelf-life of products (Kohajdova & Karovicova, 2007). During fermentation, yeast and mainly lactic acid bacteria play an important role. Lactic acid bacteria produce lactic acid that lowers the pH to 3.5-4. Proteinases perform at this pH primary proteolysis. A second proteolysis is performed by the lactic acid bacteria (Gänzle et al., 2008). This results in an increase of amino acids that is associated with in improvement in protein digestibility (Kohajdova & Karovicova, 2007). It is overall well known that a heat treatment results in denaturation of proteins. Because of the heat energy, proteins turn back to their primarily structure. During this process no peptide bonds are broken so the sequence of amino acids remains the same (Lodish et al., 2013).

In Ethiopia, tef flour is mainly used to make *injera* or flat bread. This fermented-circular soft bread is very popular in Ethiopia and forms the traditional basic diet (Tatham et al., 1995). A flowchart for the preparation of *injera* is given in figure 2.5. There are two main steps during the process. The first step involves a fermentation. The fermentation process starts after adding the backslope. The fermentation step takes about 24-72 hours at 25°C. The second step involves a baking step which is normally performed for 2 to 3 minutes at about 200 to 250ºC (Wrigley et al., 2016).

Injera is different from other types of bread because of its high moisture content and its chewy and elastic properties. The flour can also be used to prepare porridge, gruel (*muk*), homemade beverages and several gluten-free food preparations like cakes (The National Academies, 1996).

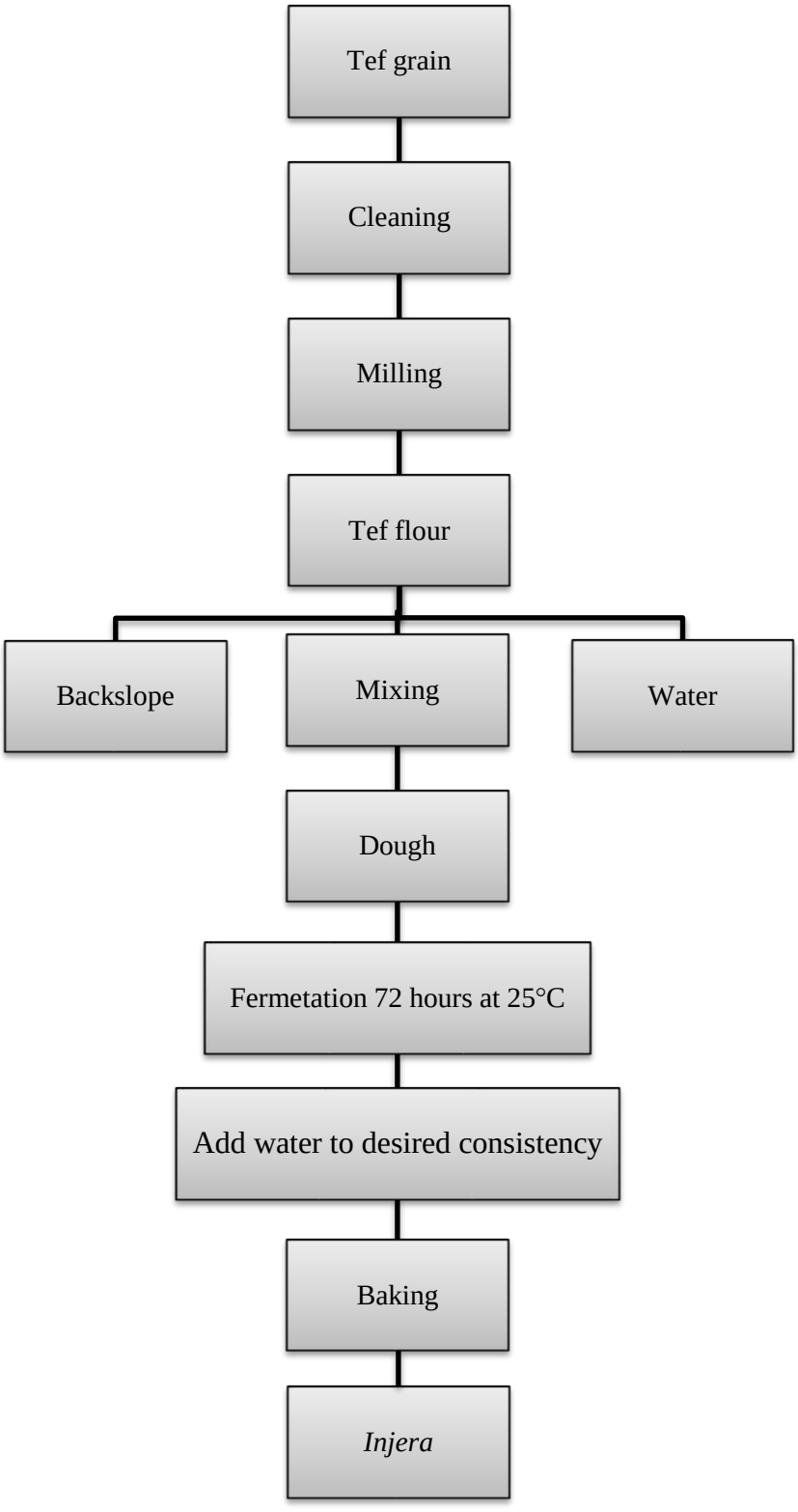


Figure 2.5: Flowchart for *injera* preparation (Wrigley et al., 2016)

2.2 Food proteins

Proteins in food are known for their nutritional value, functionality in foods and in some cases for their harmful effects. All biological protein sources are described as food proteins. The most important protein sources in the human diet include milk, meat, eggs, cereals, legumes and oilseeds. To be described as food proteins, there are some terms: nontoxic, nutritional value, digestibility, functionality in foods and bioavailability. The nutritional value depends on the amino acid composition of the protein. If proteins contain a lot of essential amino acids, the biological value of the protein is high. Now-a-days, proteins are also used in the food industry because of their effects on texture, color and flavor (Ustunol, 2015).

In European adolescents, the total protein intake is 96 gram per day. From this amount, 58 grams originates from animals while the intake of plant proteins is only 38 gram per day (Lin et al., 2015). For human nutrition all around the world, plant proteins are mostly derived from soybeans, canola, corn, wheat, rice, sorghum and millet. It should be noted that the protein content and composition of the proteins are influenced by growth conditions, cultivation and processing (Ustunol, 2015).

2.2.1 Protein content

Different researches show that the crude protein content of tef is comparable to that of many other cereals (Mulugeta, 1978). The normal tef protein content ranges, according to Wrigley et al. (2016), from 9.4 to 13.3 g per 100g flour (N x 6.25) on a dry weight-basis. Also Adebowale et al. (2011) found a protein content in white Ethiopian tef of 10.2 ± 0.9 g/100g flour (N x 6.25) on dry weight-basis. Bultosa (2007) found a grain protein content ranging from 8.7 to 11.1 g/100g (N x 6.25) in 13 different tef varieties. A comparative study with other cereals is shown in table 2.2.

Table 2.2: A comparative study of crude protein content in different cereals[1]

	Protein content (g/100g)	Substance	DW/FW	Conversion factor	Source
Tef	9.4-13.3 9.3-11.1 8.7-11.1	Flour Flour Grain	DW DW DW	N x 6.25 N x 6.25 N x 6.25	Wrigley et al., 2016 Adebowale et al., 2011 Bultosa, 2007
Amaranth	16.2-16.8	Flour	DW	N x 5.85	Alvarez-Jubete et al., 2010
Barley	12.3-16	Flour	DW	N x 6.25	Yu et al., 2016
Buckwheat	12.2-12.8	Flour	DW	N x 5.7	Alvarez-Jubete et al., 2010
Maize	4.5-9.87	Flour	FW	N x 6.25	Enyisi et al., 2014
Millet: **Finger** **Foxtail** **Pearl**	 6.7-12.4 11.6 12.21-12.99	 Whole grain Whole grain Flour	 DW FW DW	 N x 6.25 N x 5.7 N x 6.25	 Vadivoo et al., 1998 Petr et al., 2003 Chowdhury & Punia, 2006
Oats	9.5-13	Flour	DW	N x 5.4	Klose & Arendt, 2012
Quinoa	14.2-14.8	Flour	DW	N x 5.96	Alvarez-Jubete et al., 2010
Rye	8.26-8.34	Whole grain	DW	N x 6.25	Nilsson & Aman, 1997
Wheat	8.7-12	Flour	FW	N x 6.25	Kieffer et al., 1996

2.2.2 Amino acid composition

Amino acids are classified into two groups: nutritionally essential and nutritionally nonessential. Among the essential amino acids belong those that must be provided by the diet (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine). Nonessential amino acids are those whose carbon skeletons can be synthesized in the human body (Wu, 2010). The most common amino acids in tef are glutamic acid, alanine, proline, aspartic acid, leucine and valine. Compared to the other cereals the amino acid composition in tef is slightly different (Table 2.3). The amino acid composition of tef is given according to two different researches. Remarkably the content of three essential amino acids namely methionine, phenylalanine and histidine are higher than in other cereals. The content of serine and glycine, on the other hand, are much lower than in other cereals (Wrigley et al., 2016).

[1] DW or dry weight; FW or fresh weight

Table 2.3: Comparative study of amino acid composition of different cereals[2]

	Tef[a]	Tef[b]	Amaranth[c]	Barley[d]	Buckwheat[e]	Maize[d]	Millet[e]	Oats[e]	Quinoa[f]	Rye[d]	Wheat[d]
Alanine	10.1	10.1	5.97	5.1	4.63	11.2	11.2	6.7	5.53	6	4.3
Arginine	4.5	5.15	4.73	3.3	9.91	2.4	3.1	5.4	9.71	3.7	2.8
Asparagine + aspartic acid	6.4	6.4	ND	4.9	10.2	5.9	7.7	8.1	10.54	6.9	4.2
Cysteine	1.8	2.5	0.53	1.5	2.73	1.6	1.2	2.6	1.39	1.6	1.8
Glutamine + Glutamic acid	21.8	21.8	ND	24.8	17.6	17.7	17.1	19.5	17.29	23.6	31.1
Glycine	3.1	3.1	6.15	6	6.09	4.9	5.7	8.2	6.26	7	6.1
Histidine	2.8	3.21	1.99	1.8	2.47	2.2	2.1	2	4.09	1.9	1.8
Isoleucine	4	4.07	3.78	3.7	3.93	3.6	3.9	4	3.02	3.6	3.8
Leucine	8.1	8.53	7.46	6.8	6.92	14.1	9.6	7.6	6.88	6.6	6.8
Lysine	3	3.68	5.92	2.6	5.84	1.4	2.5	3.3	6.30	3.1	1.8
Methionine	3.3	4.06	0.27	1.6	1.41	1.8	2.9	1.7	2.27	1.3	1.4
Phenylalanine	5	5.69	4.49	4.3	4.62	4	4	4.4	4.52	3.9	3.8
Proline	8.2	8.2	4.53	14.3	4.45	10.8	7.5	6.2	3.54	12.2	12.6
Serine	4.1	4.1	5.4	6	5.02	6.4	6.6	6.6	5.62	6.4	6.6
Threonine	3.6	4.32	4.86	3.8	3.71	3.7	4.5	3.9	4.41	4	3.2
Tryptophan	1.3	1.3	ND	0.7	ND	0.2	1	0.8	ND	0.5	0.7
Tyrosine	3	3.84	3.65	2.7	2.7	3.1	2.7	2.8	3.66	2.2	2.3
Valine	5.9	5.46	4.43	6.1	5.23	5	6.7	6.2	3.67	5.5	4.9

[a] g/100g of total protein of the grain (DW) (Wrigley et al., 2016)
[b] g/100g of total protein of the grain (DW) (Gebremariam et al., 2014)
[c] g/100g of total protein of the grain (DW) (Andini et al., 2013)
[d] g/100g of total protein of flours (FW) (Gobbetti & Ganzle, 2013)
[e] g/100g of total protein of flours (DW) (Bonafaccia et al., 2003)
[f] g/100g of total protein of flours (FW) (Ranhotra et al., 1993)

[2] DW or dry weight; FW or fresh weight

2.2.3 Protein digestibility

Cereals provide an important amount of proteins in human nutrition. Therefore, it is important to know the protein digestibility of cereals. Because in-vivo experiments are often expensive and time taking, in-vitro experiments are mostly chosen (Dahlin & Lorenz, 1993). There are several in vitro techniques possible varying by using one or more enzymes. The pH-drop method of Hsu et al. (1977) using three different enzymes (protease-trypsin-chymotrypsin or peptidase-trypsin-chymotrypsin) is most often used as an estimation of in vitro protein digestibility (Tinus et al., 2012). The pH-drop is caused by the protein degradation resulting in a release of amino acids, peptides and especially protons (Moyano et al., 2015; Tinus et al., 2012). The IVPD of different cereals are given in table 2.4. The starting material and used enzymes are influencing factors of the IVPD and are for this reason also listed in table 2.4. The IVPD of tef is not included in this table because no results are found in literature. There is a big difference in IVPD between the different cereals. The IVPD of wheat was found the be the highest while those of barley was the lowest.

Table 2.4: A comparative study of IVPD of different cereals[3]

	IVPD (%)	DW/FW	Substance	Enzymes	Source
Amaranth	72.4-76.8	FW	Flour	Trypsin Chymotrypsin Peptidase	Bejosano & Corke, 1998
Barley	28-29.1	DW	Flour	Trypsin Chemotropism Peptidase	Sher et al., 2011
Buckwheat	65-78.8	DW	Grain	Pepsin Pancreatin	Dogra, & Awasthi, 2015
Finger Millet	41.1-54.1	FW	Flour	Pepsin	Antony & Chandra, 1998
Maize	65.3-67.9	FW	Flour	Pepsin	Duodu et al., 2002
Oats	58.39	FW	Groat	Pepsin Trypsin	Li & Xu, 2015
Pearl Millet	60.8-71.7	DW	Flour	Pepsin Pancreatin	Chowdhury & Punia, 2006
Quinoa	77.29-79.46	FW	Flour	Trypsin Chymotrypsin Peptidase	Elohaimy et al., 2015
Rye	83	DW	Flour	Pepsin Pancreatin	Aura et al., 1999
Wheat	86.4	DW	Flour	Trypsin Chymotrypsin Peptidase	Abdel-Aal & Hucle, 2002

[3] DW or dry weight; FW or fresh weight

To calculate the IVPD (%) of proteins, the technique is mostly used as a one-point pH-measurement after 10 minutes (Eq. 2.1). This formula can be re-written as equilibrium 2.2 by using pH 8 as a starting point. By converting this formula again, the end equilibrium 2.3 can be used as a difference in pH after a time period of 10 minutes. This last formula is most often used because it starts from the exact starting pH that is around pH 8 but not exactly pH 8 (Tinus et al., 2012).

$$IVPD = 210.46 - 18.10pH_{10min} \quad \text{(Eq. 2.1)}$$

$$IVPD = 210.46 - 18.10\ (8- \Delta pH_{10min}) \quad \text{(Eq. 2.2)}$$

$$IVPD = 65.66 + 18.10\Delta pH_{10min} \quad \text{(Eq. 2.3)}$$

2.2.4 Protein fractions

Proteins play a key role in the ability of the cereals to be processed into different foods. Proteins are usually classified according to their solubility. This classification is based on the Osborne extraction procedure dividing the cereal proteins into four groups: albumins, globulins, prolamins and glutelins (Zilic et al., 2011). These four groups are again divided into the non-gluten proteins and gluten proteins. Gluten are proteins present in several cereals to which sensitive people respond with specific reactions. The non-gluten proteins in cereals consist mainly of water-soluble albumins and salt-soluble globulins (Singh et al., 2001). Gluten proteins on the other hand, consists of ethanol-soluble monomeric prolamins and acid-soluble polymeric glutelins (Zilic et al., 2011).

The content of the four protein fractions in tef according to three different researches are given in table 2.5. The results of the first two researches are comparative, while the results of Adebowale et al. (2011) are totally different. The differences may be attributed to the use of different protocols. Depending on the level of the salt extractions, temperature, time and many other parameters, the protein content of the different fractions varies (Janssen et al., 2016). These values can be compared with the content of the four protein fractions of other cereals (Table 2.6). In the most common types of cereals like barley, maize and wheat, prolamins and glutelins are the dominant protein fractions. The most abundant Osborn fractions in pseudocereals like buckwheat and quinoa, are albumins an globulins.

Table 2.5: The content of the Osborn fractions in tef according to three different researches

	g/100g protein (Wrigley et al., 2015)	g/100g protein (Mulugeta, 1978)	g/100g protein (Adebowale et al., 2011)
Albumin	24-39	36.6	9.1-11.1
Globulin	7-34	11.8	
Prolamin	3-15	6.7	37.4-39.4
Glutelin	28-42	44.55	13.6-26.2

Table 2.6: A comparative study of the content of the Osborn fractions in different cereals

	Amaranth flour[a]	Barley grain[b]	Buckwheat flour[c]	Maize flour[f]	Finger millet flour[d]	Foxtail millet grain[e]	Pearl millet flour[g]	Oats grain[b]	Quinoa grain[e]	Rye flour[h]	Wheat flour[f]
Albumin	26.4	3-5	43.8	4	12.1	12.8	22-28	1-12	64.3	26	9
Globulin	25	10-20	7.82	2				50-80			5
Prolamin	5.81	35-45	10.5	55	42.6	38.7	22-35	4-15	4.71	65	40
Glutelin	42.7	35-45	14.6	29	24	9.93	28-32	<10	18.4	9	46
Rest	0.09	≤ 17	23.28	10	21.3	38.57	5-28	≤ 45	12.59	/	/

[a] g/100g protein (Srivastava & Roy, 2011)
[b] g/100g protein (Klose & Arendt, 2012)
[c] g/100g protein (Guo & Yao, 2006)
[d] g/100g protein (McDonough et al., 2000)
[e] g/100g protein (Petr et al., 2003)
[f] g/100g protein (Kumagai, 2010)
[g] g/100g protein (Nambiar et al., 2011)
[h] g/100g protein (Gellrich et al., 2003)

2.2.4.1 Non-gluten proteins: albumin and globulin

According to Wrigley et al. (2016), tef is different from the most common cereals (eg. wheat, barley, rye…) in having higher amounts of high digestible albumins and globulins (Table 2.5). Because of this, tef is presumed to have a high protein digestibility compared to other cereals (Wrigley et al., 2016). This characteristic is influencing the functionality of the grains. Different compositions in protein fractions such as albumins and globulins result in differences in baking performance and dough rheology (Adebowale et al., 2011). Albumins and globulins have mostly structural and metabolic functions. They include enzymes like α- and β-amylase, α- and β-amylase inhibitors and lipid transfer proteins (LTP's) (Verhoeckx et al., 2015). For both albumins and globulins, the molecular weights are mostly lower than 25 kDa because of their monomeric form. Sometimes the molecular weight can also vary from 60-70 kDa when they occur as polymers (Malik, 2009; Gupta et al., 1996). Both protein fractions have a high nutritional value because they contain high amounts of lysine and methionine (Malik, 2009).

2S albumin is a seed storage protein, defined on the basis of their sedimentation coefficient. They are used by the plant as a source of nutrients like amino acids and carbon structures. Specific characterisations of this family are the presence of a skeleton of eight cysteine residues and a three-dimensional structure enriched in alfa-helices. 2S albumin has a high stability and contains a high amount of sulphur-containing amino acids like cysteine and methionine. More specific, because of this characteristic, albumin is related to the prolamin superfamily. These proteins are described in many researches as major food allergens in seeds of many plants (Moreno & Clemente, 2008).

Globulins are salt-soluble seed storage proteins that mostly have a sedimentation coefficient of seven. The 7S globulins can be found in protein bodies in the embryo and aleurone layer of the grain. They are not essential for the grain but only function as storage proteins (Shewry & Halford, 2002). 7S globulins (M_r 150 000-190 000 Da) are trimeric proteins that have no cysteine residues. Therefore, they cannot form disulphide bonds. The subunit compositions are very variable because of post-translational processing (proteolysis and glycosylation) (Shewry et al, 1995). The molecular weight of the subunits range from 40-80 kDa (Breiteneder & Radauer, 2004). The 11-12S storage globulins are located in the endosperm but only occur in some cereal grains like oats (Shewry & Halford, 2002; Shewry et al, 1995). The 11S storage globulins consist out of six subunit pairs interacting noncovalent. Each subunit pair is built up out of an acidic subunit (M_r 40 000 Da) and a basic subunit (M_r 20 000 Da) linked together by a disulphide bond. 7S and 11S globulin fractions show both low amounts of cysteine and methionine in their structure. The amount of these amino acids present in 11S globulins are mostly little bit higher than in 7S globulins (Shewry et al, 1995).

2.2.4.2 Gluten proteins: prolamin and glutelin

Gluten proteins consist of two main protein groups namely monomeric prolamins and polymeric glutelins. Both consist of high repetitive amino acid sequence of glutamine and proline. The only difference can be found in the size and sequence of this repeated sequence (Verhoeckx et al., 2015). The easiest way to make a difference between glutelins and prolamins is on the basis of their solubility. Prolamins are soluble in alcohol while glutelins are insoluble because of their polymeric structure. After reduction of the disulphide bonds, they also become soluble in alcohols (Wieser, 2007).

Prolamins are known as the main storage proteins in most cereals (Shewry & Tatham, 1990; Zhang et al., 2016). These storage proteins in cereals have an important nutritional value for humans and animals (Zhang et al., 2016). Prolamins can be up to 50% of the total amount of proteins in some cereals. They contain a high amount of proline and amide nitrogen derived from glutamine (Shewry & Tatham, 1990). In tef, there are three different types of prolamins, namely α/β-, γ- and δ-prolamins. The molecular mass of these prolamins varies from 10 to 50 kDa (Zhang et al., 2016). The α/β- and γ-prolamins consists of a N-terminal domain containing repetitive sequences of glutamine and proline. The C-terminal end has no repetitive structures but contains intramolecular disulphide bonds because of the presence of cysteine residues. The δ-prolamins have no cysteine residues so they cannot form disulphide bonds. The sequence of these proteins consists out of repetitive motives (Verhoeckx et al., 2015). For most cereals, the prolamins get trivial names based on their Latin generic names (Shewry & Tatham, 1990). In case of tef, the prolamins are called eragrostins originating from *Eragrostis* tef (Zhang et al. 2016). The amount of these eragrostins is very variable depending on the solvent type used during the extraction (Table 2.5) (Wrigley et al., 2016). In wheat, the prolamins are called gliadins (Shewry & Tatham, 1990).

Glutelin polymers are built up of single polypeptides linked through disulphide bonds. This ethanol-insoluble fraction of gluten in wheat is called glutenin. In all other types of cereals, they are called as glutelins (Shewry & Tatham, 1990). They are among the largest protein molecules in nature. According to reduced SDS-PAGE, glutelins are divided into two groups according to their molecular weight: high molecular weight (100-140 kDa) and low molecular weight (30-55 kDa) (Zilic et al., 2011). The LMW-glutenins are sulphur-rich and have the same structure as the α/β- and γ- prolamins (Verhoeckx et al., 2015).

2.3 Gluten

Gluten are proteins found in several cereals such as wheat, barley and rye. Gluten are composed of two major components namely prolamin and glutelin. They also contain a small amount of lipids. In the last decade, three main health problems occurred because of gluten in the humans diet: allergy, intolerance and coeliac disease. For these patients, reactions can be avoided by a lifetime gluten-free diet or by avoiding the food products they respond too (Spaenij-Dekking et al., 2005).

The European Commission has set rules about the composition and labelling of food products suitable for people responding on gluten. Food products can be labeled as "gluten-free" (not exceeding 20 mg/kg), "very low gluten" (not exceeding 100 mg/kg) and “gluten containing” (exceeding 100 mg/kg) (The European Commission, 2009).

2.3.1 Allergy

2.3.1.1 Allergens and allergic reactions

The prevalence of food allergies has increased in the last couples of decades (Kumar et al., 2013). Several causes underlie this augmentation. For example, the introduction of enzymes into detergents is known as an important cause of increased susceptibility to allergens (Huby et al., 2000). The European law (number 8513; 2009, August 5) declares there are 14 important allergens that have to be indicated on product labels (Fig. 2.6) (Hoge gezondheidsraad, 2009).

Figure 2.6: Food containing allergens (Hampshire County Council, 2016; FAVV, 2016)

Allergens are proteins capable of inducing an allergic response in susceptible individuals. The degree of response depends on individuals varying from mild symptoms like erythema or rhinitis to acute, possibly fatal anaphylactic shock. In order to have an allergic response, the proteins should contain specific epitopes recognized by the immune system. Allergens must have B-cell epitopes to which immunoglobulin IgE can bind. T-cell epitopes on the other hand are necessary to induce a T-lymphocyte response (Huby et al., 2000).

The immunology response proceeds in two main phases. At first contact called sensitization, macrophages phagocytes present food allergens. Those macrophages present the antigenic peptides on its surface facilitating the physical interaction with T-cells. T-helper cells pass the collected information of the allergen onto a B-lymphocyte. As a response to this information, B-lymphocytes converts into plasma cells capable of producing IgE-immunoglobulins. Next IgE-immunoglobulins binds on the

specific receptors present on mast cells and basophils as shown in figure 2.7 (Kumar et al., 2013). At second contact with the allergens, the mast cells and basophils release allergic mediators like histamine, prostaglandins, leukotrienes and other inflammatory mediators. These agents cause the specific symptoms of the allergenic reaction (Huby et al., 2000).

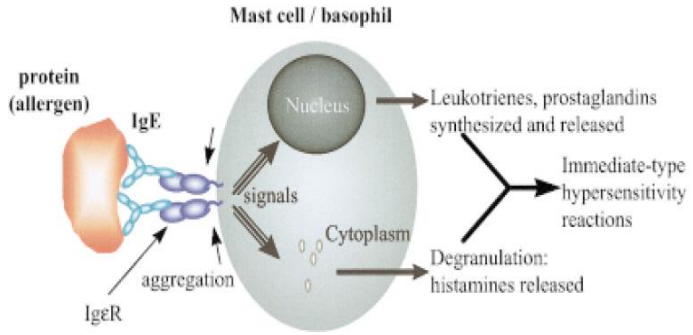


Figure 2.7: Mechanism of an allergic reaction (Huby et al., 2000)

2.3.1.2 Allergen classification

An allergen classification system is set by the International Union of Immunological Societies and World Health Organisation based on the food source. Overall, they can also be classified into groups based on their structure and functional properties. Plant food allergens can be divided according to figure 2.8. Not all plant allergens are also important for cereals. Therefore, the allergen groups important for the cereals are shortly described (Breiteneder & Radauer, 2004; WHO/IUIS Allergen Nomenclature Sub-Committee, n.d).

The cupin superfamily are seed storage proteins. These globulins can be divided into 7S vicilin-type globulins and 11S legumin-type globulins. The composition of these two groups is given in section 2.2.4.1. Next, the prolamin superfamily consists out of a skeleton of eight cysteine residues and they have a low molecular weight. Their name is originated from the major seed storage proteins in cereals, the prolamins. Now they are expanded with several plant allergen families. 2S albumins are already described in section 2.2.4.1. The nonspecific lipid transfer proteins (nsLTPs) consists out of 7-9 kDa monomeric proteins held together by four disulphide bonds forming a hydrophobic tunnel. In cereals they are especially found in corn. Worldwide, losses of crops can be assigned to insects that use plant tissues as food. Plants developed a natural resistance to this problem by producing defense compounds and proteins. Examples of such enzymes are the cereal α-amylase and protease inhibitors. The most important cereals able to produce these enzymes are wheat, barley and rye. The subunits consists of 120-160 amino acid residues. They occur as monomers, dimers or tetramers held together by disulphide bonds. The last group of the prolamin superfamily are the cereal prolamins themselves. Cereal prolamins consist of two protein fractions, the glutelins and the prolamins (Breiteneder & Radauer, 2004). IgE-binding experiments especially gave reactions with the LMW-glutelins and the linear epitopes of the repetitive domains of the α/β- and γ-prolamins (Verhoeckx et al., 2015). The δ-5-prolamins of wheat are allergens that mainly occur in allergic reactions in young children (Breiteneder & Radauer, 2004).

Under the plant defense system three important protein families are incorporated: pathogenesis-related proteins (PRs), proteases and protease inhibitors. The PRs are produced by plants as a response on infections of pathogens like fungi, bacteria, viruses and environmental factors. The proteins do not form a superfamily but are a collection of unrelated protein families that are part of the plant defense system. PR-9 are peroxidases that perform an important role in cereals. These lignin-forming enzymes use H_2O_2 in oxidative reactions. Also nsLTP's are part of the PRs and can therefore also be noted as PR-14. The protease inhibitors are described before (Breiteneder & Radauer, 2004).

Profilins are important structural proteins in some cereals. These proteins play an important role during cytokinesis, cytoplasmic streaming, cell elongation, growth of pollen rubes and root hairs. They are involved in these processes because of their ability to interact with microfilaments. The profilins have a molecular weight ranging from 12 to 15 kDa. Mostly they are allergens originating from plant pollen (Breiteneder & Radauer, 2004). Finally, a lot of enzymes are also involved in allergic reactions (WHO/IUIS Allergen Nomenclature Sub-Committee, n.d).

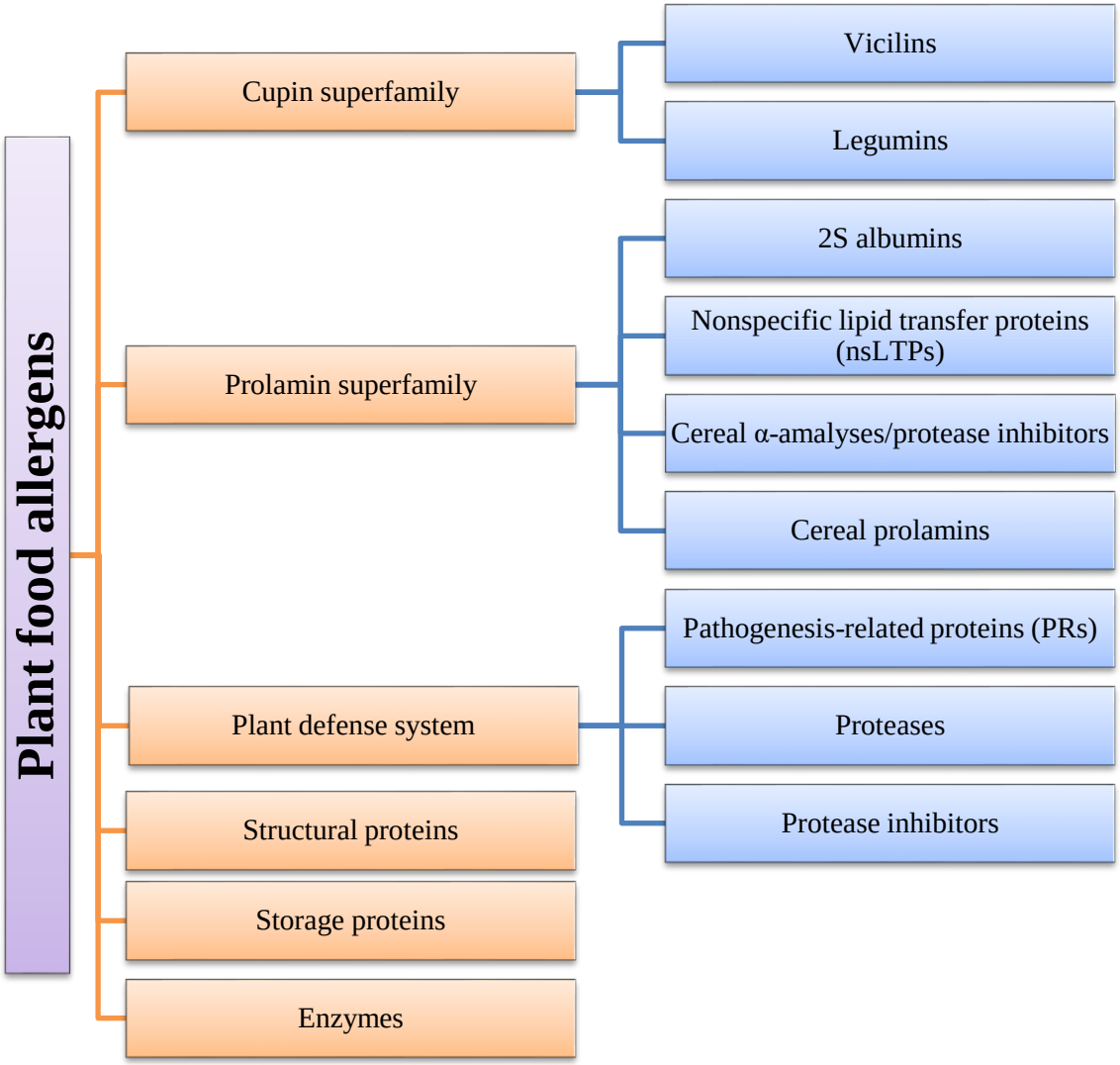


Figure 2.8: Plant food allergens (Breiteneder & Radauer, 2004)

2.3.1.3 Allergens in different types of cereals

The allergens in different cereals are given in appendix 1. Tef and quinoa are not mentioned in this table because no allergens of these cereals are reported in literature.

In amaranthus species only two allergens have yet been detected. These are no food allergens but are deriving from amaranthus pollen (Appendix 1). In western countries, there is a great interest in this type of cereals because they are presumed to be gluten-free (Wallner & Hofer, 2015). Although, a clinic-immunologic evaluation, performed by Kasera et al. (2013), rejects these conclusions. A susceptible patient was tested on allergic reaction by immunoblotting. This resulted in 16 protein fractions (13-72 kDa) able to bind with IgE-antibodies. To fully guaranty the presence of allergens in amaranthus, further research is required (Wallner & Hofer, 2015).

In barley, a mixture of food allergens and no food allergens are present. Barley is one of the four most important cereals in the world next to wheat, maize and rice. It is often used in baby food, soup, and several alcoholic drinks like beer and whiskey (Steinman, 2012). As described in table 2.6, barley has a high glutelin and prolamin content.

Buckwheat is a staple cereal in a lot of Asian societies. The main protein fraction in buckwheat is albumin (Table 2.6). All allergens detected in buckwheat are known as food allergens. The 2S albumins in buckwheat belong to the prolamin superfamily. These storage proteins collected in protein bodies, are used as a source of nutrients during germination. 2S albumins are very stable allergens able to sensitize allergic people directly in the gastro-intestinal route. Also other important allergic groups like vicilins from the cupin superfamily and α-amylase/trypsin inhibitors from the prolamin superfamily are possible allergens in buckwheat (Wallner & Hofer, 2015).

There are several species of millet. Allergens are mainly found in foxtail millet. Only a few allergens are detected in common millet and no allergens are yet detected in two other species: Japanese millet and pearl millet (Allergome, 2015). In overall, allergic reactions to millet are very rare. Nevertheless, the enzyme starch synthase can lead to an allergic reaction by inhalation and by ingestion depending on the type of cereal (Rombold et al., 2008).

Maize or corn is known to contain several allergens arriving from pollen, seed and leafs (Allergome, 2015). The main Osborn fractions in maize are the gluten proteins prolamin an glutelin (Table 2.6). According to Pastorello et al. (2000), the major food allergen in maize is a 9 kDa protein called lipid transfer protein.

Allergens are detected in two different oat varieties: *Avena sativa* and *Trisetum paniceum*. Allergic reactions are mainly deriving from the pollen. There are only two allergens in the seeds namely 11S globulin and a glutelin (Allergome, 2015).

Finally, wheat contains more allergens than all the other cereals. It is also one of the most cultivated cereals all over the world. For this reason, it is well known for its allergenic perspectives. They are able to sensitize people by inhalation leading to diseases like bakers' asthma. The main cause of this reactions are the alfa-amylase inhibitors. The second form of allergy is caused by ingestion of the allergens leading to anaphylaxis. Especially parts of the gluten proteins (δ5-gliadins) are responsible for this reaction. The other important allergens are nsLTPs, gluten and chloroform/methanol soluble proteins (CMs) (Tatham & Shewry, 2008).

2.3.1.4 Allergens and food processing

Cereals are consumed by humans after processing varying from cooking to complex fermentation processes. The effect of the processing on the cereal proteins can result in a reduction, no change or an increase in the number of epitopes present in proteins (Tatham & Shewry, 2008). In general, heat destroys epitopes resulting in a decrease of protein allergy. Only in a few cases, heat leads to a formation of neo-epitopes causing an increase of allergic reaction (Shin et al., 2012). For example, the amylase/trypsin inhibitor family in wheat maintained their IgE-binding capacity even after heating. Wheat LTP-structures, present in albumin/globulin fractions, are less stable resulting in the loss of their structure after heat treatment. Because of this, IgE is not able to bind to LTP anymore. In case of fermentation, lactic acid bacteria produce lactic acid lowering the pH. This makes cereal protease able to hydrolyze possible toxic peptides (Verhoeckx et al., 2015; Gänzle et al., 2008).

2.3.2 Intolerances

Intolerances are caused by errors in the human metabolism resulting in negative reactions of the body. In general, intolerances exist because of malabsorption of some agents as a result of the deficiency/absence of particular enzymes, hormones or other important factors. In case of gluten intolerance, the specific digestive enzyme, peptidase is absent (FAVV, 2016; Rostami et al., 2015). In contrast with allergens, symptoms of intolerances occur much more slowly (Huby et al., 2000).

2.3.3 Celiac disease

Celiac disease (CD) is a lifetime gluten-sensitive autoimmune disease of the small intestine resulting from environmental (gluten) and genetic factors (human leukocyte antigen (HLA)) (Gujral et al., 2012). It is a gluten intolerance affecting about 1% of the population in Europe, North and South America, North Africa and the Indian subcontinent (Tatham & Shewry, 2008). People with diabetes or other autoimmune disorders have a higher risk for the development of celiac disease. The most important symptoms are gastrointestinal-related such as malabsorption, diarrhoea, steatorrhea (fat in feces) or no symptoms at all (Gujral et al., 2012).

Especially the prolamin fraction in gluten is responsible for the immune reaction in CD. Prolamin has a high glutamine content and specific sequence patterns which makes prolamins resistant to gastrointestinal proteolytic enzymes. This results in an incomplete gastrointestinal digestion of gluten (Gujral et al., 2012).

According to Spaenij-Dekking et al. (2005), no gluten or gluten homologues were present in different tef varieties. Those results indicate the suitability of tef in the diet of patients with celiac disease. Although in vivo tests should be performed to completely guaranty the safety of tef for these patients (Spaenij-Dekking et al., 2005). An epidemiological study conducted in The Netherlands (2006), questioned and tested 7990 members of the Dutch Celiac Disease Society on the development of symptoms after the consumption of tef. The survey of the first round showed that 15% of the people consuming tef had complaints. During the second round, 84.5% of the people where CD-patients of which 66% are tef consumers. Only 17% of those people had complaints after the intake of tef. Out of these results, it was concluded that a wide amount of CD-patients can consume tef without having symptoms. The reduction of symptoms, in this research, was attributed to the reduction in gluten intake or an increased fibre intake (Hopman et al., 2008).

3 Material and method

3.1 Sample description

All experiments were performed with tef flour derived from Axum Agricultural Research Center (Tigray, Ethiopia). Before harvest, the cereals were dried on the field and milled into flour by a local tef miller in the same way as done for local food products. One kilogram of each variety was pre-milled and discarded to avoid cross contamination among them. The flours were stored at -18°C until use. Seven varieties of tef were tested: Boset, Dega, Quncho, Simada, Tsedey, Zagurey and Zezew. The first five are white varieties while Zagurey and Zezew are brown. The moisture content of the flours ranged from 7.9-8.4 g/100g of flour. Flour of all seven varieties were used to analyze crude protein content, Osborn fractionation, gluten determination, IVPD and SDS-PAGE. *Injera* was prepared out of four different varieties: Quncho, Tsedey, Zagurey and Zezew and tested for IVPD and SDS-PAGE. *Injera* was prepared according to figure 2.5. The proteins of the fermented dough were also analyzed with SDS-PAGE.

All of these experiments gave an indication of the protein characterization of tef and its suitability as a gluten-free flour. All information obtained during this research is important for later incorporation of tef in food preparations. This research gave information about the suitability of tef as replacement of gluten-containing cereals like wheat especially for gluten allergic, intolerant and celiac disease patients.

3.2 Statistical analyses

All results are statistically analysed with ONE-WAY ANOVA with exception from IVPD of *injera* that is analysed with TWO-WAY ANOVA. The results are given as an average and their standard deviations. For each experiment the p-values are given.

3.3 Particle size

3.3.1 Principle

Tef grains were milled into flour by a local tef miller in Ethiopia. Milling is meant to reduce the particle size (Nguyen et al., 2015). This leads to an increase of surface area and disruption of internal structures (Tinus et al., 2012).

3.3.2 Materials

All used materials for the determination of the particle size are given in table 3.1.

Table 3.1: Material particle size

Equipment
Sieve shaker
Balance
Sieves with different pore size: 850 µm 425 µm 300 µm 212 µm 150 µm

3.3.3 Method

Mass of each empty sieve was weighed in advance. The stacks of sieves were placed on the shaker. Thirty grams of flour was placed on the top sieve. After 10 minutes of shaking, the mass of each sieve was noted. From this, the mass of the flour left on each sieve was calculated.

3.4 Crude protein content (ISO 937-1978)

3.4.1 Principle

The Kjeldahl method is a standard procedure for determining nitrogen. There are three main steps in this process. The first step is a digestion according to reaction 3.1.

$$\text{Sample} + H_2SO_4 + \text{Kjeldahl tablet} \rightarrow (NH_4)_2SO_4 + CO_2 + SO_2 + H_2O \qquad \text{(r 3.1)}$$

The second step involves a distillation according to reaction 3.2 and 3.3.

$$(NH_4)_2SO_4 + 2\ NaOH \rightarrow Na_2SO_4 + 2\ H_2O + 2\ NH_3 \qquad \text{(r 3.2)}$$

$$H_3BO_3 + H_2O + NH_3 \rightarrow NH_4^+ + B(OH)_4^- \qquad \text{(r 3.3)}$$

The last step is a quantification of ammonia (r. 3.4). The content of ammonia is proportional to the nitrogen content in the sample (Munoz-Huerta et al., 2013). The most common method is a titration with 0.1 N HCl and phenolphthalein as an indicator until the color turns back to purple. Based on equilibrium 3.1, the amount of nitrogen present in the grain can be calculated (International Organization for Standardization, 1978). The amount of protein in dry flour can be calculated from the N-content using the conversion factor 5.4 (Mariotti et al., 2008).

$$NH_4^+ + B(OH)_4^- + HCl \rightarrow NH_4Cl + HB(OH)_4 \qquad \text{(r 3.4)}$$

$$N\ (\%) = \frac{V_{HCl}\ x\ N_{HCl}\ x\ MM_N\ x\ 100}{Fresh\ weight\ sample\ x\ 1000} \qquad \text{(Eq. 3.1)}$$

3.4.2 Material

All equipment and products used during the Kjeldahl experiment are listed in table 3.2.

Table 3.2: Material Kjeldahl method

Equipment
Analytical balance
Destruction tubes 6x
Destruction chamber
Distillation unit
Burette
Erlenmeyer's 6x
Pipet 20 mL

Products
Kjeldahl tablet composed of: 235 g Na_2SO_4 4 g $CuSO_4.5H_2O$ 5 g selenium powder Sulphuric acid 95-97% Phenolphthalein Tashiro solution (1 L) 10 g boric acid 0.2 L ethanol 10 mL methylred 2 mL methylblue Filled to 1 L by distilled water NaOH 32% HCl 0.1 N

3.4.3 Method

One gram of flour was weighed in a destruction tube. Then, 20 mL of concentrated sulphuric acid and a Kjeldahl tablet were added to each destruction tube. The mixtures were heated in a destruction chamber for approximately three hours. During this incubation time, the mixtures turned into clear green solutions (r 3.1). After cooling down, 50 mL of distilled water and three drops of phenolphthalein indicator were added.

The distillation unit was cleaned with water before use. A destruction tube was placed in the distillation unit and 32% of NaOH was added until the mixture turned blue (r 3.2). A flask with 25 mL of Tashiro solution was placed at the outlet of the distillation unit. Each sample was distilled for about four minutes. During this step, the distillate was collected into the flask with Tashiro solution that changed color from purple to green (r 3.3). Afterwards, the distillation unit was again rinsed with water.

In the last step of the Kjeldhal method, the flask with distillate was titrated against 0.1 N HCl until the color turned back to purple (r 3.4). The amount of protein was calculated (Eq. 3.1).

3.5 Osborn fractionation

3.5.1 Principle

The Osborn method was used for the fractionation of albumin, globulin, prolamin and glutelin on the basis of their solubility. Albumins are soluble in water, globulins in NaCl-solution, prolamins in ethanol and the glutelins in acetic acid. The amount of each protein fraction was determined by the Kjeldahl method, as described in 3.4. The amount of protein fraction was calculated according to equilibrium 3.2 (Zilic et al., 2011).

$$\% \text{ protein fraction} = \frac{(V_{HCl} - V_{Blank}) x\ N_{HCl}\ x\ MM_N\ x\ 5.4\ x100\ x100}{Dry\ weight\ sample\ x\ total\ protein\ content\ x\ 1000} \qquad \text{(Eq. 3.2)}$$

3.5.2 Material

All material and products used during the Osborn method are listed in table 3.3.

Table 3.3: Material Osborn method

Equipment
Analytical balance Magnetic stirrer Magnets Centrifuge Falcon tubes Dialysis tubes (MWCO 12400 Da) ±35 cm Rotavapor
Products
NaCl-solution 0.5 M Ethanol 70% Acetic acid-solution 0.05 M

3.5.3 Method

The whole procedure was performed at 4°C and all centrifugation steps took place at 1500 rpm for 30 minutes at 4°C. In a falcon tube, 10 g of flour was weighed, followed by the addition of 40 mL of 0.5 M NaCl. This mixture was stirred for two hours at 4°C. The tubes were centrifuged and the supernatant was decanted into a collection bottle. Again 40 mL of 0.5 M NaCl was added, this time for one hour. After centrifugation, the supernatant was decanted in the same collection bottle. Next, 40 mL of distilled water was added and stirred for 30 minutes. After centrifugation, the supernatant was pooled and decanted in the same bottle as in the previous two steps. These supernatants were dialyzed against distilled water for 48 hours to remove salt. The dialyzed samples were centrifuged, to separate the globulin fraction in the pellet from the albumins in the supernatant.

The residue left after extraction with the salt solution was used further for the separation of the prolamin and glutelin fraction. The residue was extracted with 40 mL of 70% ethanol by stirring for 2 hours at 4°C. After centrifugation, the supernatant, containing the prolamin fraction was decanted. This step was repeated with 40 mL of 70% ethanol for 1 hour. The supernatants were pooled in a bottle. The prolamin fractions were evaporated at 55°C for about 20 minutes with a rotavapor to remove the ethanol.

In the last step of the Osborn method, the residue obtained after extracting the prolamins, was extracted with 40 mL of 0.05 M acetic acid for 2 hours under continuous stirring at 4°C. After centrifugation, this step was repeated for 1 hour. The supernatants were pooled into a bottle.

The four protein fractions were freeze-dried for about 72 hours. The amount of the four protein fractions were determined by the Kjeldahl method with a destruction time of 2 hours. The percentage of the protein fractions were calculated using equilibrium 3.2.

3.6 Gluten determination

3.6.1 Principle

A commercial ELISA-test kit was used to measure the content of gluten-like proteins in the tef flours. The test is based on a direct sandwich-ELISA (Fig. 3.1). A microtiter plate was coated with monoclonal R5-antibodies able to bind with potentially toxic QQPFP pentapeptide found in prolamin fractions. After the binding of the prolamin sample, a second antibody marked with an enzyme was added. This enzyme is capable of changing the substrate. After adding the stop solution, this substrate was spectrophotometrically measured at 450nm (R-Biopharm AG, 2012).

The concentration of gluten was determined using a standard curve, simultaneously runned with the sample. For calculating the amount of gluten, a cubic spline function was used. In order to obtain the correct gluten concentration, the dilution factor was included and the results were multiplied by two. This correction factor was necessary because prolamins usually represents 50% of the proteins present in gluten (Ballabio et al., 2011; R-Biopharm AG, 2012).

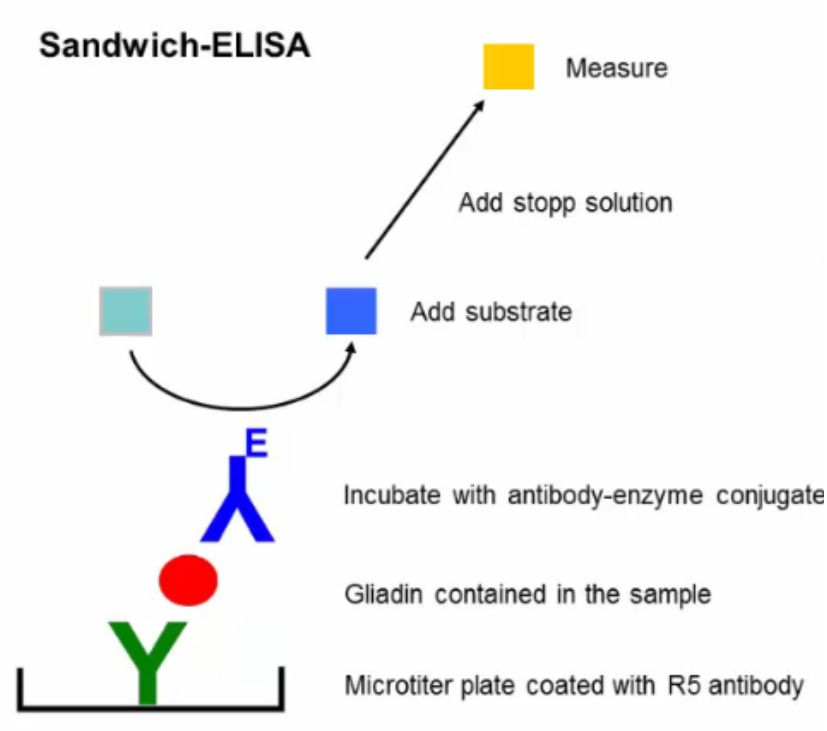


Figure 3.1: Sandwich-ELISA (R-biopharm AG, 2016)

3.6.2 Materials

All equipment and products used during the gluten determination are given in table 3.4.

Table 3.4: Material ELISA

Equipment
RIDASCREEN® Gliadin (Art. No. R7001) Microtiter plate coated with monoclonal R5 antibodies Micropipette 1000 µL Micropipette 200 µL Multichannel pipette 200 µL Hood Falcon tubes 50 mL Waterbath at 50°C Shaker Centrifuge
Products
RIDASCREEN® Gliadin (Art. No. R7001) D.1 Buffer (5x) (concentrate)-sample dilution D.2 Conjugate (11x) D.3 Buffer (washing) concentrate (10x) Substrate Chromogen Stop-solution Standard curve (0, 5, 10, 20, 40 and 80 ng/mL) Ethanol 80% Cocktail patented solution (Art. No. R7006)

3.6.3 Method

Before start, the chromogen solution was checked if it had a bluish color. Because the chromogen is light-sensitive, it was covered with aluminium. During the whole process, everything was cleaned with 60% of ethanol to avoid cross-contamination.

The test was performed on all seven varieties of tef. Commercial wheat and gluten-free flours purchased from a local supermarket were used as positive and negative controls. For the preparation of the samples, a cocktail patented solution was necessary. Of each variety, 0.25 g of flour was weighed into a falcon tube. Then, 2.5 mL of the cocktail patented solution was added to the falcon tube and vortex mixed until the flour was completely dissolved. This suspension was incubated for 40 minutes in a waterbath at 50°C. After cooling until room temperature, 7.5 mL of 80% ethanol was added and the suspension was vortexed. This was then shaken for one hour. Every 10 minutes the suspension was extra shaken manually to increase the efficiency of the extraction. After centrifugation at 2000 rpm for 10 minutes at room temperature, the supernatant was transferred to a clean falcon tube.

The supernatant was then diluted 1:12.5 with sample dilution and vortex mixed. Then, 100 µL of the diluted sample was immediately transferred to the microtiter plate. Each sample was performed in duplicate. Also, 100 µL of the standards were transferred to the microtiter plate in duplicate. The plate was gently beaten from one side to make sure that the liquid made contact with the inside of the well. This was then incubated for 30 minutes at room temperature. The content in the wells was then discarded and the microtiter plate was gently tabbed upside-down on a paper to completely remove the content. Then, 250 µL of washing buffer was added to each well and also removed in the same way as the previous step. This step was repeated two more times. After this washing step, 100 µL of diluted conjugate was added to each well. After gentle beating, the plate was incubated for 30 minutes at room temperature. When the content was removed out of the wells, the washing step was again repeated three times. Next, a mixture of 50 µL of substrate and 50 µL of chromogen was added to each well. After gentle beating, the plate was incubated for 30 minutes at room temperature in the dark. Finally, 100 µL of stop solution was added to each well. Within 10 minutes, the absorbance was measured at 450 nm. The gluten content was calculated with the cubic spline function according to the users' manual.

3.7 SDS-PAGE

3.7.1 Principle

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the most commonly used method to separate protein molecules based on their size at high resolution. Proteins were first denatured with an anionic detergent (SDS). SDS binds to the proteins in order to give all proteins a negative charge proportional to their molecular mass. Then, the proteins are separated on the basis of their molecular mass by electrophoresis through a porous acrylamide gel matrix. The negatively charged proteins move at a speed inversely proportional to their size to the anode (positively charged). Proteins with a high molecular mass migrate slower than those with a low molecular mass. Finally, the separated proteins are made visible by a specific staining solution (Nowakowski et al., 2014).

3.7.2 Materials

All material necessary to perform SDS-PAGE are listed in table 3.5.

Table 3.5: Material SDS-PAGE

Equipment
Micropipettes Magnetic stirrer Centrifuge Eppendorf tubes Water cooker Freezer Criterion XT Precast Gel 12% Bis-Tris Bio-Rad Criterion Electrophorese tank Consort EV233 Power supply Shaker
Products
Extraction buffer (pH 8.8) 5 M Urea 50 mM Dithiothreitol 2% SDS 2 M thiourea 0.1 M Tris-HCl Bio-Rad XT Sample Buffer 4x; Cat. #161-0791 Bio-Rad XT MOPS running buffer 20x; Cat. #161-0788 Bio-Rad G250 (Coomassie blue); Cat. # 161-0787 Bio-Rad Molecular Weight Standard: Broad range standards Myosin 200 000 Da B-Galactosidase 116 250 Da Phosphorylase b 97 400 Da Serum albumin 66 200 Da Ovalbumin 45 000 Da Carbonic anhydrase 31 000 Da Trypsin inhibitor 21 500 Da Lysozyme 14 400 Da Aprotinin 6 500 Da

3.7.3 Method

Before the start of the SDS-PAGE, sample extraction was performed according to Nguyen et al. (2015). Flour (40 mg) was diluted by 2 mL of extraction buffer. This suspension was incubated at room temperature for 16 hours under stirring (200 rpm) conditions. Afterwards, the samples were centrifuged in Eppendorf tubes for 30 minutes at 13 000 rpm. The supernatant was stored at -18°C until analysis.

A final concentration of approximately 1 µg protein/µL was used in all cases. A specific volume of supernatant and 50 µL of XT sample buffer was brought into an Eppendorf tube. The molecular weight standard was prepared using a volume of 10 µL. Water was added until a total volume of 200 µL. This suspension was shaken for 60 minutes at ambient temperature. The Eppendorf tubes were then brought into a boiling water bath for maximum 5 minutes. The tubes were immediately cooled by putting them in the freezer.

During the cooling time, gel preparation was performed. The gel was rinsed with distilled water and the tape at the bottom of the cassette was removed. After this, the comb was carefully removed out of the gel. The gel was then placed into the slots of the tank of the Criterion cell, with the buffer chamber facing towards the center of the cell. This buffer chamber was then filled with 60 mL of 1x running buffer. After removing of possible air bubbles, the gel was loaded.

From each sample, 20 µL was loaded while only 10 µL of the standard was loaded. After filling the lower buffer chamber with 1x running buffer, the lid was placed on the tank. The electrode cables were then connected to the power supply. The power was turned on so the gel could run at 140 V for about 90 minutes. In that time the dye front had reached the bottom of the gel.

Afterwards, the power was shut down, electrodes were disconnected and the lid was removed. The cassette was then removed out of the electrophoresis tank. The running buffer was discarded out of the tank and the tank was cleaned with distilled water. By using the cassette opening tool built into the lid, the Criterion gel cassette was broken.

The gel was then stained for 60 minutes in Coomassie blue (G250) at gentle shaking. Then, the staining solution was removed and distilled water was added to destain the gel for about 15 minutes. This step was repeated until no blue color was coming off the gel anymore. Overnight, the gel was placed in distilled water at gentle shaking. During this time the bands were developed.

After a conservation step, the gels can be stored for a long time. The gel was put in a 25% (v/v) ethanol, 3% (v/v) glycerol suspension for one hour. The gel was covered in plastic. All air bubbles were removed from the gel and the gel was dried for about 12-16 hours.

To analyse the result, the logarithms of the molecular weight markers were put in function of the relative migration distance. A logarithmic function was used to calculate the molecular weight of the proteins.

The same process was repeated for the Osborn fractions of Tsedey, Quncho, Zagurey and Zezew. In order to obtain the correct final concentrations, 0.01 g of fractions were dissolved in different volumes according to their protein content. Albumins were dissolved in 800 µL of buffer, globulins in 1500 µL, prolamins in 300 µL and glutelins in 50 µL. The suspension was vortexed and shaken at ambient temperature for 16 hours. The Eppendorf tubes were then centrifuged at 13 000 rpm for 30 minutes and stored in a freezer until use. The same process for SDS-PAGE was followed as described before.

SDS-PAGE was also performed on the fermented dough and *injera*. These samples were freezedried for 72 hours and milled into flour again. This substance was then extracted the same way as the flour. The same procedure was performed to separate the proteins on the gel.

3.8 In vitro protein digestibility

3.8.1 Principle

The in-vitro digestibility of proteins in tef was assayed using a pH-drop method. For this experiment three enzymes were used: trypsin, chymotrypsin and protease (Appendix 2). These three digestive enzymes are capable of breaking down proteins resulting in a decrease of pH. The percent of in-vitro digestibility was calculated according to equilibrium 3.3 (Nguyen et al., 2015; Tinus et al., 2012; Hsu et al., 1977).

$$IVPD = 65.66 + 18.1\ \Delta pH_{10min} \quad \text{(Eq. 3.3)}$$

3.8.2 Materials

The necessary equipment and products for IVPD are given in table 3.6.

Table 3.6: Material In Vitro Protein Digestibility

Equipment
Blue cap bottles (100 mL) Waterbath at 37°C pH-analyzer Mortar
Products
NaOH 0.1 M HCl 0.1 M Trypsin from porcine pancreas 13,000-20,000 U/mg (Sigma-Aldrich T0303-1G) α-Chymotrypsin from bovine pancreas ≥40 U/mg (Sigma-Aldrich C4129-1G) Protease from *Streptmyces griseus* ≥3.5 U/mg (Sigma-Aldrich P5147-1G)

3.8.3 Method

A multi-enzyme mixture was prepared according to Nguyen et al. (2015) consisting of trypsin (1.6 mg/mL), chymotrypsin (3.1 mg/mL) and protease (1.3 mg/mL). This enzyme mixture was stored in an incubator of 37°C until use. According to the Kjeldahl results of each variety, a flour suspension of 1 mg N/mL was prepared with a total volume of 50 mL. This suspension was then shaken for one hour at 37°C in a waterbath. The flour suspension and enzyme mixture were adjusted to pH 8 by adding sodium hydroxide. Five millilitres of the multi-enzyme mixture was added to the flour suspension. The start-pH was immediately measured. The sample was then incubated in the waterbath at 37°C for 10 minutes. After this time, the end-pH was measured and the in-vitro digestibility was calculated according to equilibrium 3.3.

The same procedure was performed for *injera*. The freeze dried *injera* was milled in a mortar and a mass was taken to make a flour suspension of 1 mg N/mL. The IVPD was determined in the same way as described above.

4 Results

4.1 Particle size

There was no big difference in particle size among the flour of the different tef varieties. The particle size range of the seven tef flours were separated as: 100% < 850 µm, 99-100% < 425 µm, 96-99% < 300 µm, 78-85% < 212 µm, 66-77% < 150 µm.

4.2 Protein characterization

Seven tef varieties were analysed on their crude protein content (Fig. 4.1) and wheat was used as a control. The crude protein content of wheat was found to be 9.32 ± 0.17 g/100g flour (db) by using 6.25 as conversion factor. This result was comparable with the value announced on the package of the wheat flour (9.76 g/100g of flour (db)).

The crude protein content for tef varied from 8.48-9.44 g/100g of flour (db) and were significantly different among varieties ($p < 0.001$). Color of the grain does not affect the protein content because the results showed no clear difference in crude protein content between the two red varieties (Zagurey and Zezew) and the five white varieties.

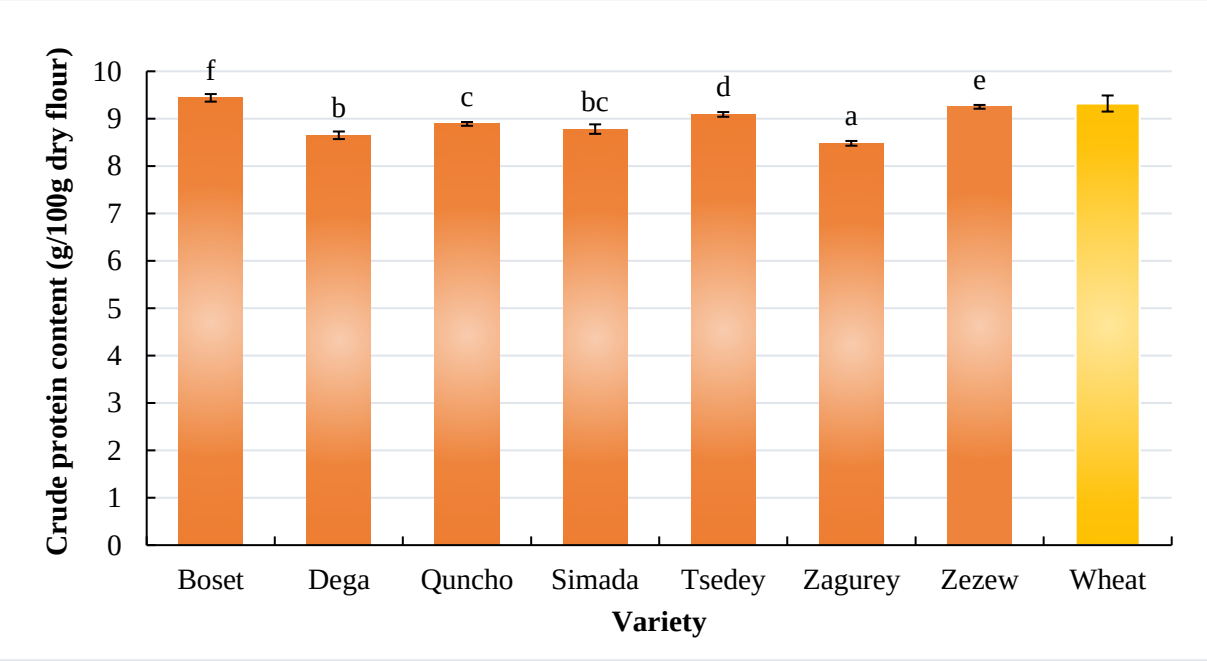


Figure 4.1: Crude protein content of tef (n=3; Tsedey: n=2) in comparison with wheat (n=3)[4]

[4] abcdef Means with a different superscript are significantly different ($p < 0.05$)

The proteins of the tef flours were separated by SDS-PAGE (Fig. 4.2). Wheat and gluten-free flour were used as a control. The molecular weights of the marker can be found next to their corresponding bands. At first sight, the protein pattern of the seven tef varieties were comparable. Only some large molecular weight proteins were absent in Dega and Zezew. Proteins in tef had a very wide range in molecular weight. The wheat protein pattern was completely different than those of the tef varieties. In wheat, high molecular weight proteins were more prominent. The protein pattern of the gluten-free flour distinguished itself from wheat and from the tef varieties in that it had smaller molecular weight proteins.

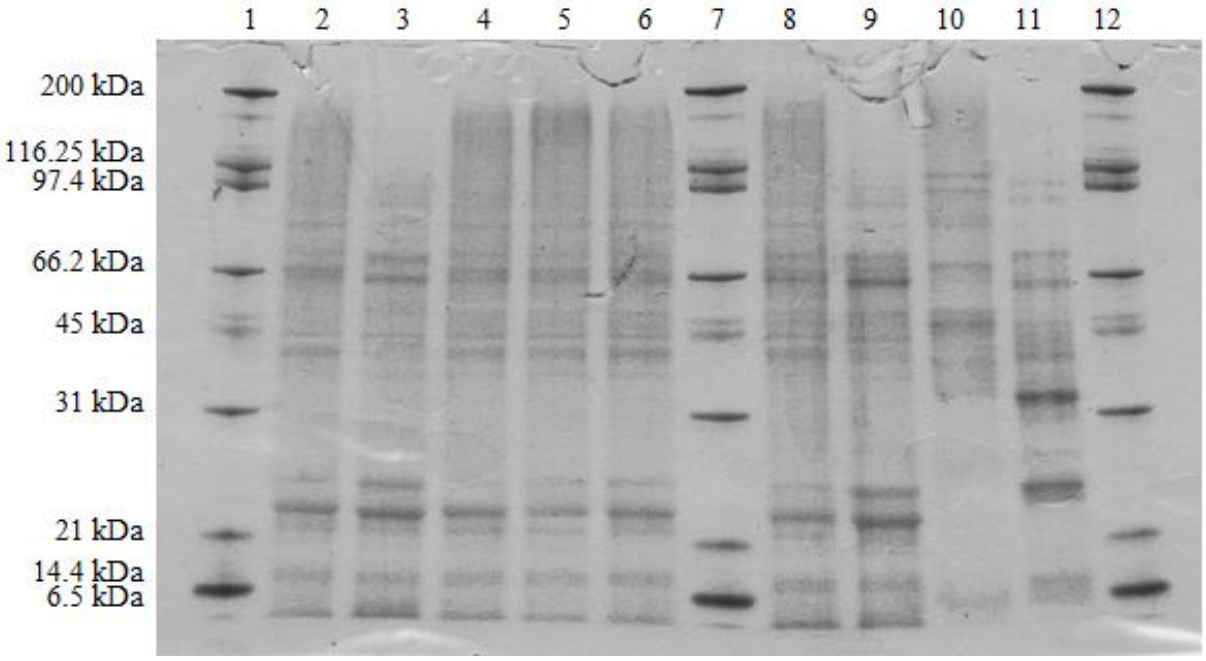


Figure 4.2: SDS-PAGE of whole tef flour compared with gluten-free flour and wheat flour as control (1,7,12: Molecular weight marker; 2: Boset; 3: Dega; 4: Quncho; 5: Simada; 6: Tsedey; 8: Zagurey; 9: Zezew; 10: Wheat; 11: Gluten-free flour)

The molecular weight of the proteins in tef, wheat, and gluten-free flour are given in table 4.1. The proteins are divided into groups based on the molecular weights of the marker. Molecular weights in bold, were more dominant proteins on the gel (darker bands). In overall, there was a big similarity between the molecular weights of the seven tef varieties. Even though, some proteins were not found in all seven varieties. Protein molecules with medium to small molecular weights were most dominant in tef. Wheat had a different protein pattern than tef because it contained more dominant bands of larger molecular weights than tef. Also, there were not that much of small molecular weight proteins in wheat as found in tef. In the gluten-free flour, no large molecular weight proteins were found. Two proteins with molecular weights around 20 kDa and 26 kDa were clearly more dominant proteins in this commercial gluten-free flour.

Table 4.1: Semi-quantitative molecular mass (Dalton) of proteins in tef, wheat and gluten-free flour

Range (Da)	Boset	Dega	Quncho	Simada	Tsedey	Zagurey	Zezew	Wheat	Gluten-free flour
>200 000	210239		210239	209437	209437	218714		219617	
116 250 - 200 000	179736	156220	179736	179245	179245	187184 162851		**187753**	
97 400 - 116 250	110177 99829	99829				104389	115132 99962		
66 200 - 97 400	91071 83575 77096 **66488**	 83575 77096 71449	91071 77096 71449	91250 77337 71710	 83789 77337 **66765**	95292 80763 69722	95292 87500 74886	**95133** **80535**	 80535 69454
45 000 - 66 200	 51560 **48721** **46145**	**62101** 54703 **48721**	**62101** 54703 51560 **46145**	**62388** 55004 51866 **46454**	58492 55004 **49029** **46454**	**61083** **51200** **46060**	61083 57440 **51200** **48511**	**64871** **60792** 57143 53860 **48203**	60792 **50894**
31 000 - 45 000	 39684 37873 36203 34657 **31890**	**43798** 39684 37873 33223	**43798** 41652 37873 36203 33223	**44107** 38179 36506 33521	 41961 38179 36506 34958 **32185**	**43819** 38123 36506 35006 32311	**43819** 38123 36506 35006 32311	 41454 37817 **36203** 34705	41454 34705 33312 32015
21 500 - 31 000	 29488 25532 23888 22421	30647 28403 26431 23134 22421	**30647** 28403 26431 24686 23888 22421	**30940** 28689 26710 24960 24158 22684	 29777 26710 24960 24158 23401	 29960 **26952** **24437** 22980	 29960 **26952** 25228 **24437** 22980	30803 27610 	30803 27610 **25788** 24166 22046

Range (Da)	Boset	Dega	Quncho	Simada	Tsedey	Zagurey	Zezew	Wheat	Gluten-free flour
14 400 - 21 500	**20496**	**19918**	**19918**	**20168**	**20168**	**20484**	**20484**		**19684**
	18843	**18343**	**18343**	**18585**	**18585**	**18906**	**18427**		19162
	17866	17411	17411	17646	17646	17969	17969		
	16975	16975		16787		17111	17531		
	15775	**15775**	**15408**	**15629**	**15629**	**15951**	**15951**		**15727**
	14717	14717	14717	14933	14933	14922	14922	15033	**15033**

4.3 Osborn fractionation

The protein content of the different Osborn fractions for the seven tef flours was determined as a proportion of the total protein content (Table 4.2). The protein fractions of the tef varieties ranged and decreased in the order of: globulin (9.55-12.93 g/100g protein) > albumin (5.43-8.68 g/100g protein) > prolamin (2.49-1.98 g/100g protein) > glutelin (0.25-0.59 g/100g protein). As tef is a gluten-free cereal, the retrieved values of the gluten proteins were very low. The albumin content revealed significant difference among the varieties ($p = 0.010$) with the highest and lowest values found for the varieties Dega and Quncho, respectively. The highest and lowest globulin fraction were found in Dega and Tsedey, respectively. The p-value ($p = 0.019$) of the globulin fractions was found to be significantly different among the varieties. The prolamin fractions among the varieties were not significantly different ($p = 0.784$). The glutelin fractions were significantly different ($p = 0.004$) with highest values for Boset and lowest values for Simada. The contents of the Osborn fractions of tef proteins were not influenced by the color of the grains.

Wheat, performed as a control, had higher amounts of gluten proteins compared with tef, prolamin (28.63 g/100g protein) and glutelin (38.57 g/100g protein). The non-gluten proteins were less prominent with 14.43 g/100g protein for albumin and 4.07 g/100g protein for globulin. All protein fractions together in wheat accounted for 85.7% of the total protein content. The recovery of tef was found to be much lower compared to wheat, and ranged between 18 and 24%.

Table 4.2: Osborn protein fractions (g/100g protein) (Tef: n=3; Zezew albumin, Simada albumin, Quncho prolamin, Wheat: n=2)[5]

	Albumin	Globulin	Prolamin	Glutelin	Recovery
Boset	6.10 ± 0.85^{a}	9.56 ± 0.96^{a}	2.04 ± 0.22	0.59 ± 0.16^{b}	18.28 ± 1.75^{a}
Dega	8.68 ± 0.42^{b}	12.93 ± 0.83^{b}	2.49 ± 0.25	0.42 ± 0.11^{ab}	24.52 ± 1.45^{b}
Quncho	5.43 ± 0.46^{a}	11.27 ± 1.89^{ab}	2.10 ± 0.82	0.27 ± 0.01^{a}	18.52 ± 0.00^{a}
Simada	6.20 ± 0.84^{a}	11.75 ± 0.56^{ab}	2.32 ± 0.24	0.25 ± 0.06^{a}	20.59 ± 1.45^{ab}
Tsedey	6.39 ± 0.52^{ab}	9.55 ± 1.22^{a}	2.11 ± 0.34	0.34 ± 0.02^{ab}	18.39 ± 1.60^{a}
Zagurey	7.30 ± 1.52^{ab}	10.76 ± 1.21^{ab}	2.13 ± 0.34	0.51 ± 0.04^{ab}	20.95 ± 3.35^{ab}
Zezew	7.19 ± 0.52^{ab}	11.88 ± 0.58^{ab}	1.98 ± 0.65	0.27 ± 0.12^{a}	21.57 ± 0.68^{ab}
p-value	**0.010**	**0.019**	**0.784**	**0.004**	**0.026**
Wheat	14.43 ± 1.14	4.07 ± 0.19	28.63 ± 4.45	38.57 ± 0.48	85.70 ± 6.25

[5] [ab] Means with a different superscript are significantly different ($p < 0.05$)

The proteins of the Osborn fractions of four tef varieties were characterized with SDS-PAGE (Fig. 4.3 and 4.4). As the protein patterns of the four Osborn fractions were found to be different, there was a good separation of the four protein fractions for all four varieties. Comparing the albumin fraction of all four tef varieties, no big difference was notable. The albumin fraction had a broad range of molecular weights. The globulin fractions were comparable in all four tef varieties and there was also a broad range of molecular weights. The high molecular weight proteins belonging to the globulins were, compared with the high molecular weight proteins of the albumins, a little bit smaller. The bands of prolamins showed similarity in all four tef varieties. There were some mediate molecular weight proteins, but most abundantly there were prolamins present with molecular weights little bit higher than those of the 21 kDa marker. Glutelins were due to their very low concentration in the Osborn fractions more difficult to visualise on the SDS-PAGE. Nevertheless, there were similarities in the glutelin pattern of Quncho, Tsedey and Zagurey. The molecular weights of the glutelins were intermediate.

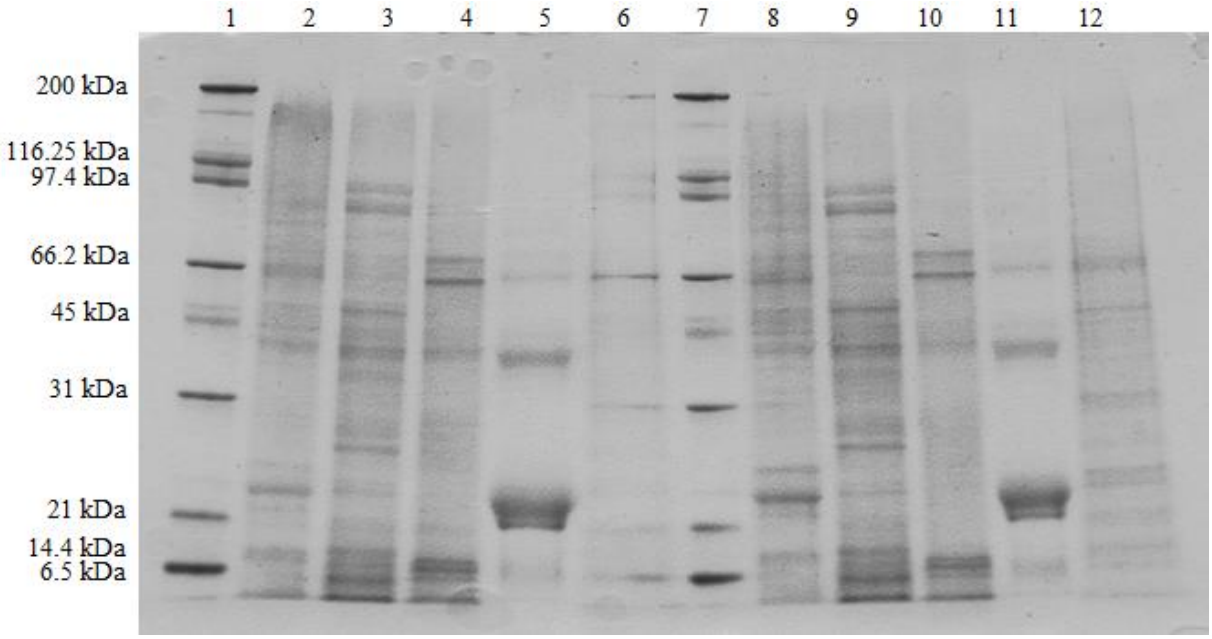


Figure 4.3: SDS-PAGE Osborn fractions Quncho and Tsedey (1,7: Molecular weight marker; 2: Quncho whole flour; 3: Quncho albumin; 4: Quncho globulin; 5: Quncho prolamin; 6: Quncho glutelin; 8: Tsedey whole flour; 9: Tsedey albumin; 10: Tsedey globulin; 11: Tsedey prolamin; 12: Tsedey glutelin)

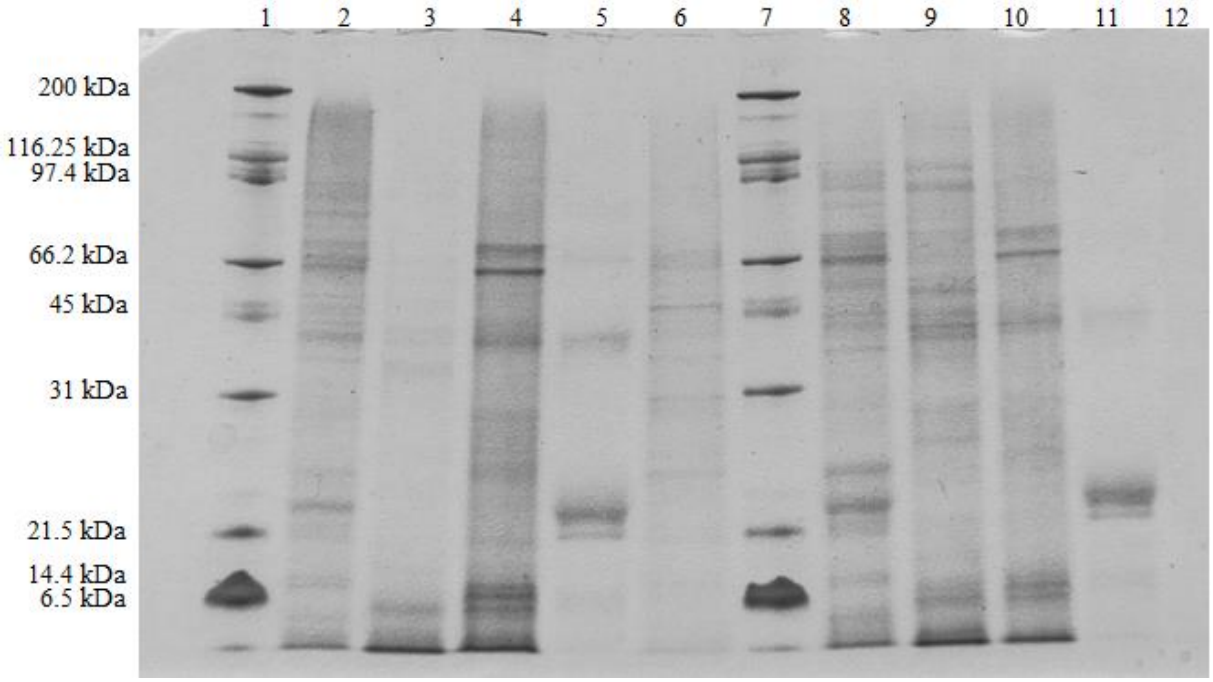


Figure 4.4: SDS-PAGE Osborn fractions Zagurey and Zezew (1,7: Molecular weight marker; 2: Zagurey whole flour; 3: Zagurey albumin; 4: Zagurey globulin; 5: Zagurey prolamin; 6: Zagurey glutelin; 8: Zezew whole flour; 9: Zezew albumin; 10: Zezew globulin; 11: Zezew prolamin; 12: Zezew glutelin)

The estimated molecular mass of proteins in each fraction is shown in table 4.3 and 4.4. The proteins are divided into groups based on their molecular weights. First of all, it should be noted that small differences could be found between the tef flour found in table 4.1 and table 4.3. Not all, or other proteins were found during the second SDS-PAGE performed on the flour, especially in the higher molecular weights. Nevertheless, the dominant proteins were comparable between the two results. Due to the loading of 1 µg/µL in each line of the gel, other proteins were found in the protein fractions that could not be determined in the whole flour.

The molecular mass of the albumins had a very broad range. Some dominant proteins had molecular weights between 66.2-97.4 kDa, 31-45 kDa and especially smaller molecular weight proteins were present, ranging between 21.5-31 kDa and 14.4-21.5 kDa.

The globulins had a broad range of molecular weight just like the albumins. As can be seen in figure 4.3 and 4.4 there were differences between the globulins and albumins. Other dominant proteins were found in the globulin fraction of tef with molecular weights ranging from 45-66.2 kDa, 31-45 kDa, and 14.4-21.5 kDa.

The prolamin fractions did not contain a lot of different molecular weight proteins. In all four tef varieties dominant proteins were found with molecular weights around 30 kDa and 17 kDa. Finally, the glutelin fraction consisted mainly of mediate and small molecular weight proteins. The number of different proteins were also for glutelin clearly lower compared to albumin and globulin. The glutelin fraction of Zezew was not good visible (figure 4.4) due to low purity of the extract. For this reason, the glutelin fraction of Zezew was not taken further into account. Some dominant glutelin proteins were found with molecular weights ranging between 45-66.2 kDa, 31-45 kDa, 21.5-31 kDa and 14.4-21.5 kDa.

Table 4.3: Semi-quantitative molecular mass (Dalton) of Osborn fractions from Quncho and Tsedey compared with their whole flour protein

Range (Da)	Quncho flour	Quncho albumin	Quncho globulin	Quncho prolamin	Quncho glutelin	Tsedey flour	Tsedey albumin	Tsedey globulin	Tsedey prolamin	Tsedey glutelin
>200 000	260908 220527			296536	303628	250853 211971				
116 250 - 200 000	165595	189731 146251	189731 165595			182325 159096	182325 140484			
97 400 - 116 250										
66 200 - 97 400	88951 81981	**88951** **70532**	88951 70532		93105 78674	93105 **78674** **67671**	**78674** **67671**	88951 70532		
45 000 - 66 200	**65787** 54369 51291 **48498**	61560 51291 48498 45954	**51291** **45954**	50923 45416	**46502**	59049 **49185**	59049 49185 46502	**51291** **45954**	53101 47367	**48498**
31 000 - 45 000	**41497** **39537** 36057 33067	**37729** 34507 **31727**	37729 36057 **33067**	32297	36162 33069	**44059** 36162 **33069**	41827 **36162** 33069	37729 36057 **33067**	33702	**37729** 36057 33067

Range (Da)	Quncho flour	Quncho albumin	Quncho globulin	Quncho prolamin	Quncho glutelin	Tsedey flour	Tsedey albumin	Tsedey globulin	Tsedey prolamin	Tsedey glutelin
21 500 - 31 000	30476	**29307**		**29690**	30401	**30401**	**30401**		**30987**	30476
	28212					28078	**28078**			
	27185									
		26220				26042				**26220**
	25312				**25116**	25116	25116			**25312**
		23648	24456			23424		24456		
		22162	22162			21916	22649	22162		22162
14 400 - 21 500	20828	20211			21223	**19944**	**21223**			**20211**
	19625	19625	19625				19352	19625		**19625**
	18536	**19067**					**18790**			
	18029					**18255**	18255		**18103**	
	17545	17545	17545	**17331**		17260			**17609**	17545
		16219	**16219**	**16856**	16796		16796	**16219**		16219
	15814	**15814**	**15814**					**15814**		
			15426			15524	**15524**	**15426**	15445	15426
		15055				**15136**	**15136**			
	14698			14783	14764		**14408**			
6 500 - 14 400						14066				

Table 4.4: Semi-quantitative molecular mass (Dalton) of Osborn fractions from Zagurey and Zezew compared with their whole flour protein

Range (Da)	Zagurey flour	Zagurey albumin	Zagurey globulin	Zagurey prolamin	Zagurey glutelin	Zezew flour	Zezew albumin	Zezew globulin	Zezew prolamin	Zezew glutelin
>200 000						201097				
116 250 -200 000						171780 149196 116904	149197	149197		
97 400 - 116 250										
66 200 - 97 400	 78533 72337		 73149 67965		 79559 73361	95119 86732 79558 67960	**86733** **73361**	**86733** **73361**		
45 000 - 66 200	 **50650** **48420**	 51219 46936	 56597 51219 45101	63218 51960 **46250**	 59026 55296 51960 **46250**	63218 55296 **51959** **46250**	 59026 55296 46250	 **51960** **46250**		 48964 46936
31 000 - 45 000	**44600** **40053** 37590 36490 33611 32769 31977	 39209 36899 33137	43431 **39209** 38012 **35863** 33988 **31580**	 **31475**	**43792** 41553 37631 **35906** 34314 31475	 **41553** 39507 37631 35905 34313	**41553** **39508** **35906** **32841** **31475**	41554 34314 **32841**	39209 **31580**	41905 40502 38012 33988 32335 31580

Range (Da)	Zagurey flour	Zagurey albumin	Zagurey globulin	Zagurey prolamin	Zagurey glutelin	Zezew flour	Zezew albumin	Zezew globulin	Zezew prolamin	Zezew glutelin
21 500 - 31 000	30523	**30865**							30865	
		30189	**30189**			30205				30189
	29220	**28939**			**29023**		29023			
	27499	**28360**								
	26978	26778	27281			26886				26778
	26005		**25837**							25837
			25396		**25011**					
	23534	24566	**24566**			24156	**24156**	24156		24566
	22498		**23436**		**23352**					
		22751	22751			22593	22593		**22751**	
	21866						**21876**	21876	**22112**	21809
	21566	21515	21515							
14 400 - 21 500	**20460**	20956	**20431**		20556			**20556**	20431	20689
	20204		**20180**			**19947**	19948		20180	
	19482	**19700**	**19700**		**19370**		19370			
				17325	17800	**17799**	17325	18298		
						16872				
				16440	16440	**16439**	15630			
				14539	14888	14887	**14539**	14888		
6 500 - 14 400					14205			**14205**		
						13575	13884	**13884**		
						12992	12993			

The Osborn fractions of wheat were also tested on the SDS-PAGE, as a control. The result of the SDS-PAGE is given in figure 4.5. Also for wheat, a good separation of the different Osborn fractions was notable. Compared to tef there was a big difference in protein pattern between the four protein fractions. The albumin and globulin pattern of wheat also had a broad range in molecular weight, nevertheless, clear differences were found between wheat and tef. The large molecular weight proteins of the albumin fraction of tef were not found in the albumin fraction of wheat. The globulins in wheat had larger molecular weight proteins than tef. The prolamin and glutenin fraction in wheat were found to be the most abundant proteins. Also in these two protein fractions, a clear difference was notable between the two cereals. The prolamins and glutelins of wheat had larger molecular weight proteins compared with tef.

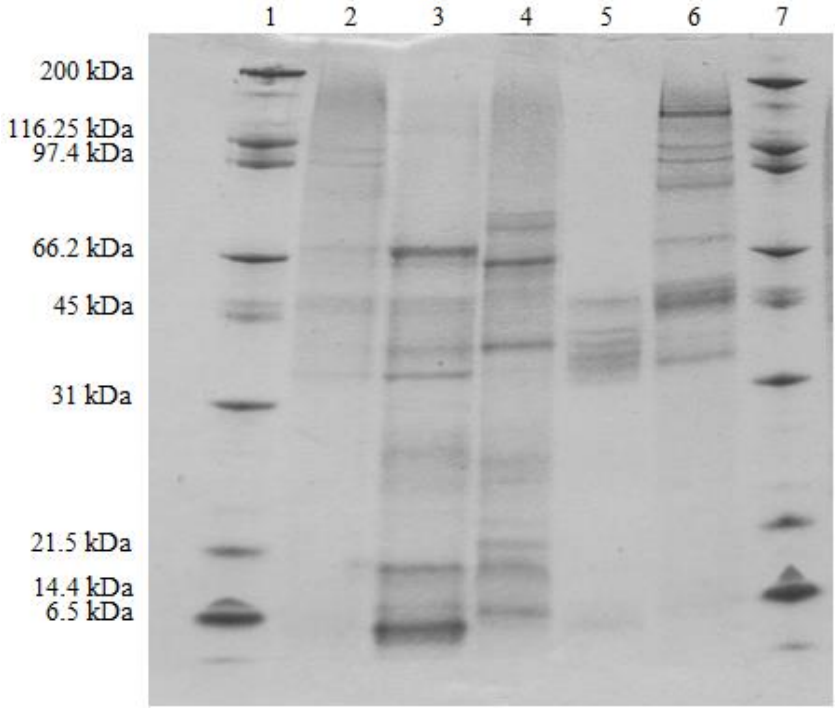


Figure 4.5: SDS-PAGE Osborn fractions wheat (1,7: Molecular weight marker; 2: Wheat whole flour; 3: Wheat albumin; 4: Wheat globulin; 5: Wheat prolamin; 6: Wheat glutelin)

The estimated molecular mass of the Osborn fractions of wheat is given in table 4.5. The molecular weights were separated based on the molecular weights of the marker. The protein pattern of the wheat flour was comparable with the protein pattern found in table 4.1. Some small differences were visible in the large molecular weight proteins. But still, the more dominant proteins were the same on both gels. Also for wheat, other proteins were visible in the protein fractions that were not visible for the flour. This could be explained, because the same concentration was loaded on the gel for the whole flour as for the Osborn fractions.

The albumin fraction contained especially mediate and small molecular weight proteins. Compared with tef, the molecular weights of wheat albumins were smaller. The dominant proteins of albumin had molecular weights around 46 kDa and between 21.5-31 kDa and 14.4-21.5 kDa. Compared with tef, the dominant proteins with high molecular weight (66.2-97.4 kDa), were not present in wheat.

The wheat globulins also had a broad range of molecular weight. The globulins of wheat consisted other proteins, with different molecular weights than tef. The proteins with molecular weights ranging between 45-66.2 kDa were all dominant proteins. Other dominant proteins were found with molecular weights around 31 kDa, 22 kDa, and between 14.4-21.5 kDa.

The protein pattern of the wheat prolamins looked similar as those found in tef although the molecular weight of the proteins were different. Also for wheat there was a small molecular weight range. The molecular weights were mediate and especially variating between 26 and 36 kDa.

Finally, the wheat glutenin fraction was completely different from the glutelin pattern found in tef. The wheat glutenins mainly consisted of large molecular weight proteins. The most dominant proteins had molecular weights between 116.25-200 kDa, 97.4-116.25 kDa, 66.2-97.4 kDa, 45-66.2 kDa, 31-45 kDa and around 29 kDa. Only a few proteins with small molecular weights were found. There were much more proteins found in the glutenin fraction of wheat compared to tef. As found during the Osborn method, the glutenin fraction of wheat was much more abundant than the glutelin fraction in tef.

Table 4.5: Semi-quantitative molecular mass (Dalton) of Osborn fractions from wheat compared with their whole flour protein

Range (Da)	Wheat flour	Wheat albumin	Wheat globulin	Wheat prolamin	Wheat glutelin
>200 000					
116 250 - 200 000	198872	128265	165419		193273 **163404**
97 400 - 116 250	**102506**		100477		**109493**
66 200 - 97 400	**84807** **71966**		83129		**88751** **74138**
45 000 - 66 200	**62263** 54698 **48649** 46061	58259 **46061**	**61031** **53615** **45149**	63344 51638	63344 58951 55077 **48566**
31 000 - 45 000	**36165**	43713 37819 36165	38831 35449 **31279**	**35409** **31016**	**39006** **37129** **35409**
21 500 - 31 000	29550 27487	**30689** **27487** 25672 22628 **21967**	28965 22180 **21532**	29763 **28599** 26500 25552	29763 **28599**
14 400 - 21 500		21341 20181 18641 **16503** 15090 **14464**	**20335** 18272 **17377** **16176** **14792** 14479		19163 14813
6 500 - 14 400	14168	13883		13541	14151

4.4 Gluten determination

The seven tef varieties were tested on their gluten content by an ELISA method. The gluten content is reported in mg/kg (db) of flour (Fig. 4.6). The gluten content in wheat, the positive control, was higher than 100 mg/kg. Commercial gluten-free flour was used as a negative control with gluten contents below 20 mg/kg. The gluten content of all tef varieties was found to be below 20 mg/kg. Boset, Zagurey and Zezew were statistically different and had a higher gluten content compared to the other four varieties ($p < 0.001$). Also these results were below 20 mg/kg, which means this is not of much relevance.

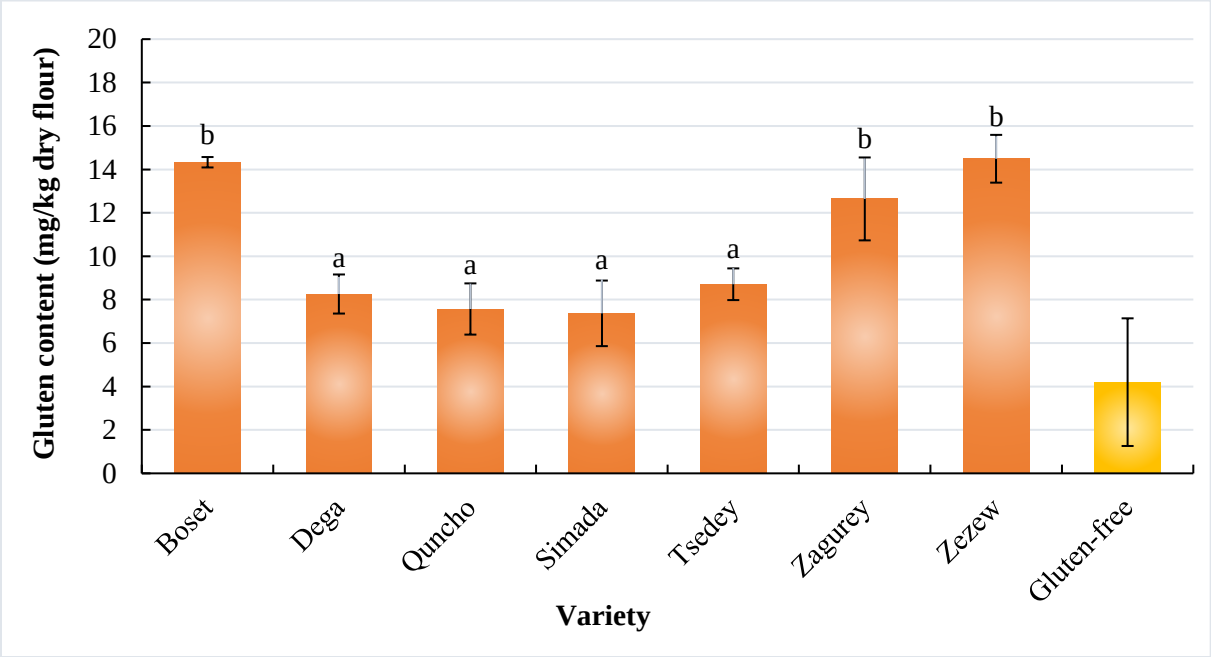


Figure 4.6: Gluten determination of tef (Boset & Zezew: n=2; Quncho, Zagurey & Gluten-free flour: n=3; Dega, Simada & Tsedey: n=4)[6]

[6] [ab] Means with a different superscript are significantly different ($p < 0.05$)

4.5 In vitro protein digestibility

The IVPD of the flour of seven tef varieties was tested by using a multi-enzyme method consisting of trypsin, chymotrypsin, and pepsin. Results of the whole flour are given in figure 4.7. There was no notable differences among the seven tef varieties and the values varied between 70.9% for Dega and 72.2% for Tsedey (p = 0.163).

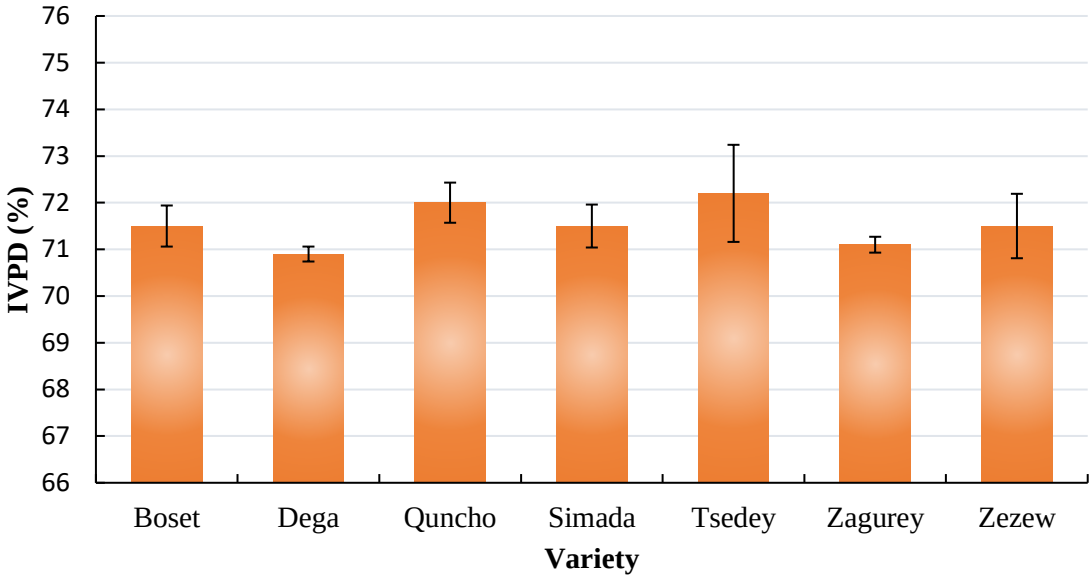


Figure 4.7: In vitro protein digestibility of tef flour (n=3)

The IVPD of *injera* prepared from four different tef varieties are given in figure 4.8 in comparison with the results of their corresponding flour. The values varied between 72.4% for Zagurey and 74.8% for Zezew. There was no interaction between the tef varieties and their *injera* preparation (p = 0.189). The IVPD from the flours was significantly lower than those from the *injera* (p = 0.001), while only a trend between varieties was observed (p = 0.056).

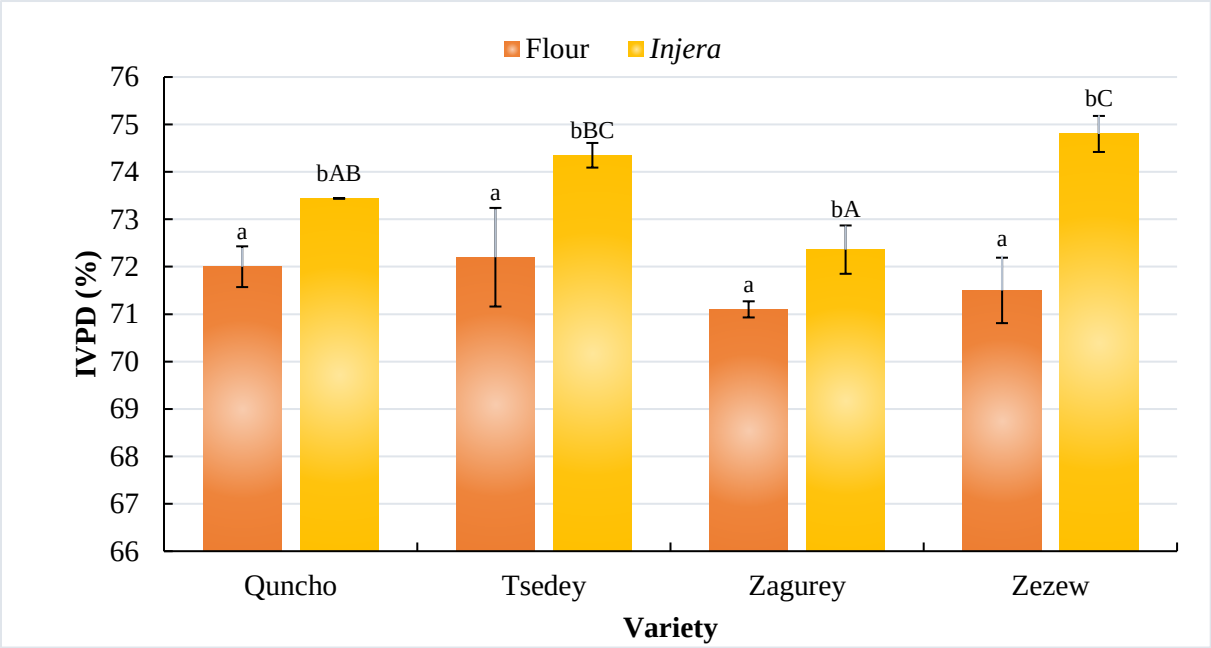


Figure 4.8: In vitro protein digestibility of tef *injera* compared to their corresponding flour (n=2)[7]

SDS-PAGE was performed for the fermented dough and *injera* of four varieties. The results of Tsedey are given in figure 4.9. The results of Quncho, Zagurey, and Zezew are given in figure 4.10. In all four cases, the main difference took place during the fermentation step. No big difference could be found between the protein pattern of the fermented dough and *injera*.

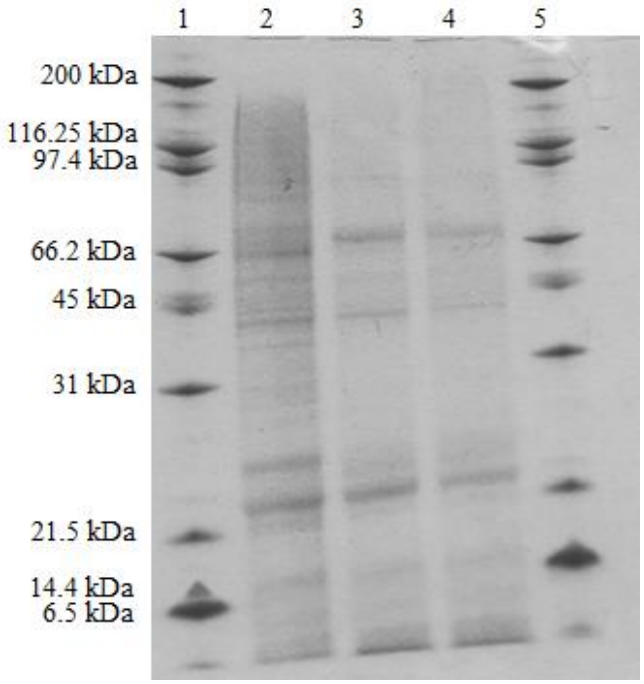


Figure 4.9: SDS-PAGE fermented dough and *injera* of Tsedey (1,5: Molecular weight marker; 2: Tsedey whole flour; 3: Tsedey fermented dough; 4: Tsedey *injera*)

[7] [ab] Bars within the same variety with a different superscript are significantly different ($p < 0.05$)
[ABC] Bars of the same color with different superscript are significantly different ($p < 0.05$)

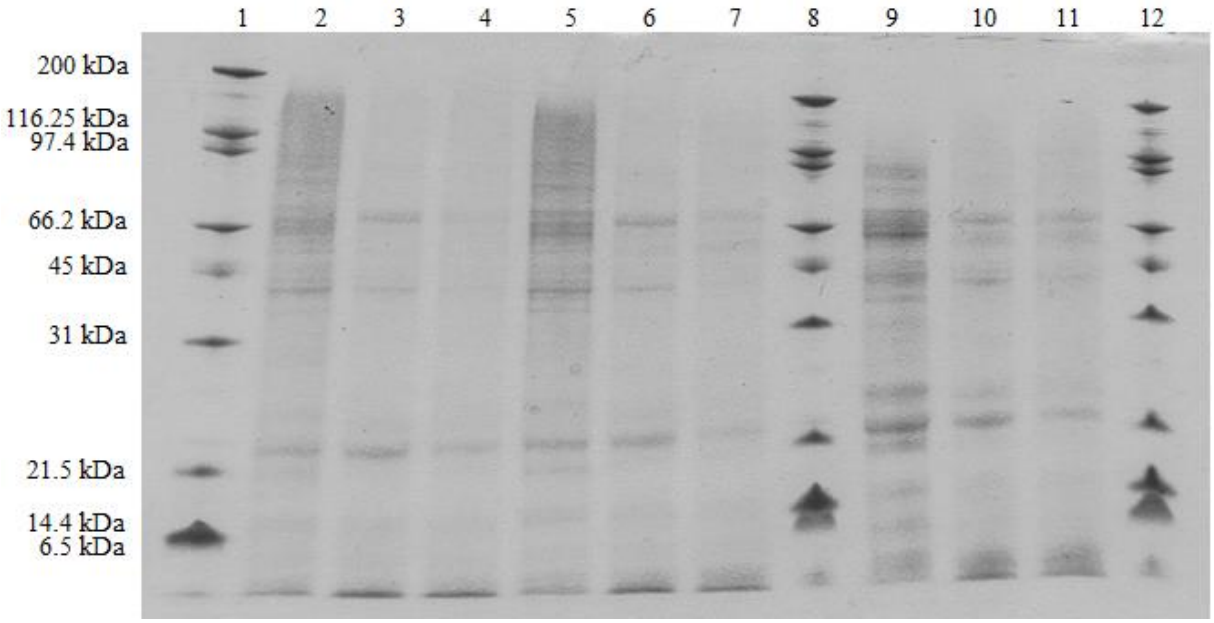


Figure 4.10: SDS-PAGE fermented dough and *injera* from Quncho, Zagurey and Zezew (1,8,12: Molecular weight markers; 2: Quncho whole flour; 3: Quncho fermented dough; 4: Quncho *injera*; 5: Zagurey whole flour; 6: Zagurey fermented dough; 7: Zagurey *injera*; 9: Zezew whole flour; 10: Zezew fermented dough; 11: Zezew *injera*)

The molecular weight of proteins in the fermented dough and the *injera* are given in table 4.6 and table 4.7. The proteins are separated based on their molecular weight. The protein patterns of the flours were comparable with the results found in table 4.1. Also, the main proteins were comparable on both gels. In all four cases, the number of proteins was reduced after the fermentation step. All proteins ranging between 21.5-31 kDa were disappeared after fermentation for all four varieties. During the baking step, the proteins were not changed. Figure 4.9 and 4.10 shows that the protein bands were changed a little bit in color. This means that the amount of proteins were possibly reduced because of the high temperature.

Table 4.6: Semi-quantitative molecular mass (Dalton) of proteins in fermented dough and *injera* prepared out of Tsedey

Range (Da)	Tsedey flour	Tsedey fermented dough	Tsedey *Injera*
>200 000			
116 250 - 200 000	185340 156697 135130 118365	124365	121601
97 400 - 116 250	104999		
66 200 - 97 400	94119 85109 77537	79905	78129
45 000 - 66 200	**65554** 60744 **52817** **49518** **46572**	**49993** 46887	48882 45845
31 000 - 45 000	39375 35605 33955 **32438**	39337 35408 **33694**	38463 36453 **34621**
21 500 - 31 000	29743 27425 25412 24503 22849		
14 400 - 21 500	**20083** **17865** **16914** 14896	19455 **17730** 16723	19022 **17868** 16831
6 500 - 14 400	14205 13570	14234 13222	14277 13244

Table 4.7: Semi-quantitative molecular mass (Dalton) of proteins in fermented dough and *injera* prepared out of Quncho, Zagurey and Zezew

Range (Da)	Quncho flour	Quncho fermented dough	Quncho *injera*	Zagurey flour	Zagurey fermented dough	Zagurey *injera*	Zezew flour	Zezew fermented dough	Zezew *injera*
>200 000									
116 250 - 200 000	189622 163298 142471							116949	116949
97 400 - 116 250	100408			108216		115531			
66 200 - 97 400	90744 82515 75441	90744	88383	96566 86806 71466	93878 84390	91221	99283 88594 79640	 81998	 81998
45 000 - 66 200	59242 **55074** **51366** 48050 45071	 **55074** 51366 45071	 **57701** 53642 46800	**60056** **55408** **51312**	 **53866** 49884 46354	 **56732** 52341 45042	59967 **55098** **50834** 47076	 **56294** 51882 48007	 **56294** 51882 48007
31 000 - 45 000	39947 37733 **35712** 32166	 37733 **35712**	 38908 **36751**	44446 38944 36591 **34459**	 **35573**	 **36789**	40777 **38119** 35729 **31615**	 38818 **36377**	 38818 **36377**
21 500 - 31 000	27829 26592 25443			30749 27642 25009 23840 21750			28211 25360 22944		
14 400 - 21 500	19980 **17931** 16205	19256 **17931** 16749	19460 **18091** 16873	**19124** **16339** **15183**	18591 **17153** 15885	**18836** **17344** 16032	**19955** **16844** **15570**	**19495** **17221** 16557	**19495** **17221** 16557
6 500 - 14 400	13873 12726	13873 12726	13922 12395	**12805** 11304	12862 11683	12922 11715	**13444** **11748** **10690** **10371**	13787 12469 11005	13787 12469 11005

4.6 Possible allergens

Table 4.8 consists possible allergens in tef, fermented dough/*injera* and wheat. This table is based on the molecular weights of allergens in different cereals (Appendix 1) and the SDS-PAGE results found during the experiments. All allergens in tef and wheat with a molecular weight of known allergic proteins and the protein fraction they belong too are listed in table 4.8. It should be noted that the Osborn fraction they belong too was not found for each allergen. The proteins are separated in the table based on their molecular weight. Next to the protein fraction, the cereals they are already officially detected in, are mentioned. This table only gives an indication of possible allergens. To completely confirm their presence in tef more experiments are required.

Because of the worldwide use of wheat in food products, their allergens are well-studied. In this research not all allergens of wheat were detected during SDS-PAGE. Those that were not found on the gels are described as X'. Especially in the high molecular weight proteins differences were found in molecular weight during SDS-PAGE of the four tef varieties. For this reason, complete exclusion of certain allergens was not always possible. If the allergen occurs in less than two varieties the allergen is described as (X).

Table 4.8: Possible allergens in tef flour, tef fermented dough/*injera* and wheat[8][9][10]

Range (Da)	Allergen	Protein fraction	Cereal	Molecular weight (kDa)	Tef flour	Tef fermented dough/*injera*	Wheat
66 200 - 97 400	HMW glutenin	Glutelin	Wheat	88			**X**
	Starch synthase	Glutelin[a]	Wheat	85-91			**X**
45 000 - 66 200	Endochitinase	Albumin/globulin[f]	Wheat	67	(X)		X'
	Purothionin	Albumin/globulin[b]	Wheat	66			X'
	Lipid transfer protein	Albumin/globulin[c]	Wheat	66			X'
	Germin-like protein	Gloubulin[g]	Wheat	65			X'
	Omega-5 gliadin	Prolamin	Wheat	65			X'
	Bèta-amylase	Albumin/globulin[c]	Wheat	60	X		**X**
	Purple acid phosphatase	Albumin/globulin[h]	Wheat	60	X		**X**
	Globulin-like protein	Globulin	Maize	49.89	**X**	X	
	Alpha-amylase	Albumin/globulin[c]	Barley	47.8	(X)	X	
	LMW glutenin GluB3-23	Glutelin	Wheat	46	**X**	X	X'
31 000 - 45 000	Serpin	Albumin/globulin[b]	Wheat	43			X
	Peroxidase	Albumin/globulin[d]	Wheat	38.8	**X**	X	X
	Omega2_Gliadin	Prolamin	Wheat	37.96			X'
	Glyceraldehyde-3-phosphate-dehydrogenase	Albumin[e]	Wheat	37	X	X	X
	Peroxidase	Albumin/globulin[d]	Wheat	36	**X**	**X**	X
	Starch synthase	Glutelin[a]	Wheat	36	X	**X**	**X**
	Grasses group 43	/	Wheat	35.64	X	X	X
	Endosperm transfer cell-specific protein	Glutelin[j]	Wheat	35.53	(X)	X	X
	Glutenin subunit	Glutelin	Maize	34.6	(X)	X	
	Elongation factor 1	Albumin/globulin[h]	Wheat	33.55	X		X'
	Dehydrin	Albumin/globulin[h]	Wheat	33.4	X		X'

[8] Allergens described as **X** are found in large concentrations during SDS-PAGE
[9] Allergens of tef described as (X) are found in maximal two tef varieties
[10] Allergens of wheat described as X' are not found during SDS-PAGE

Range (Da)	Allergen	Protein fraction	Cereal	Molecular weight (kDa)	Tef flour	Tef fermented dough/*injera*	Wheat
	Thioredoxin	Albumin/globulin[f]	Wheat	33.2	X		X'
	Gamma-hordein 3	Prolamin[f]	Barley	33.1	X		
31 000 - 45 000	Alpha-bèta-gliadin	Prolamin	Wheat	33	X		X'
	Gamma-gliadin	Prolamin	Wheat	32.6	X		X'
	Grasses group 42	/	Wheat	31.46	X		X
21 500 - 31 000	Glutenin subunit	Glutenin	Wheat	30	X		X
	Thaumatin-like protein	Albumin/globulin[f]	Wheat	29.6	**X**		X
	Chitinase	Albumin/globulin[f]	Maize, Wheat	28.98-30	**X**		X
	Thiol reductase homologue	/	Wheat	27	X		X
	Triosephosphate-isomerase	Albumin/globulin[h]	Wheat	27	(X)		**X**
	Glutenin	Glutelin	Oat	25	**X**		
	Peroxiredoxine	Albumin/globulin[h]	Barley, Maize, Rye, Wheat	23-24	X		X
	13S/11S Globulin	Globulin	Buckwheat, Oat	23-24	X		
	Proteasome subunit	Albumin/globulin[h]	Maize	23.07	X		
	Gliadin	Prolamin	Wheat	23			X'
	Peroxidase 1	Albumin/globulin[d]	Wheat	23	X		X
14 400 - 21 500	Agglutinin isolectin 1	Globulin[ij]	Wheat	21.24			X
	NFKB 1-like protein	Albumin/globulin[h]	Wheat	20.25	X		**X**
	Alpha-amylase/subtilisin inhibitor	Albumin/globulin[c]	Wheat	19.6	**X**	**X**	**X**
	7S Vicilin	Globulin[f]	Buckwheat	19	X	X	
	2S albumin	Albumin	Buckwheat	15	**X**		
	Alpha-amylase inhibitor	Albumin/globulin[c]	Barley, Rye, Wheat	15-16.43	**X**	X	**X**
	Trypsin inhibitor	Albumin/globulin[c]	Barley, Maize, Wheat	15.78-16.14	**X**	X	**X**
	Purothionin	Albumin/globulin[b]	Barley, Rye	15	**X**		
	Leucine-rich repeat protein	/	Wheat	15	X		X
	Xylanase inhibitor	Albumin/globulin[f]	Wheat	15	X		**X**
	Glutenin subunit	Glutelin	Wheat	14			X
	Thioredoxin	Albumin/globulin[f]	Maize	13-14	**X**	X	
	Profilin	Globulin[j]	Wheat	14	X	X	X
	Lipid transfer protein	Albumin/globulin[c]	Wheat	14	X	X	**X**

Range (Da)	Allergen	Protein fraction	Cereal	Molecular weight (kDa)	Tef flour	Tef fermented dough/*injera*	Wheat
6 500 - 14 400	Alpha-amylase/trypsin inhibitor	Albumin/globulin[c]	Wheat	13.9			**X**
	Ribosomal inactivating protein		Wheat	13			X
	Alpha-amylase inhibitor 0.19	Albumin/globulin[c]	Wheat	13.34			X
	Alpha-purothionin	Albumin/globulin[b]	Wheat	12			X’
	Dehydrin	Albumin/globulin[h]	Wheat	12			X’
	Alpha-amylse/trypsin inhibitor	Albumin/globulin[c]	Wheat	10			X’
	Lipid transfer protein	Albumin/globulin[c]	Barley,Wheat	9			X’
	Serine protease inhibitor	Albumin/globulin[f]	Wheat	9			X’
	Peroxidase	Albumin/globulin[d]	Wheat	9			X’

[a] Takumi et al., 2000
[b] Malik, 2009
[c] Verhoeckx et al., 2015
[d] Zilic et al., 2011
[e] Roy et al., 2009
[f] Lim, 2015
[g] Shutov et al., 2003
[h] Hurkman et al., 2009
[I] Golde et al., 1970
[j] Spizzirri & Cirillo, 2017

5 Discussion

During this research, a protein characterization of tef was performed. First, the crude protein content of tef found during the experiments was compared with the result found by Wrigley et al. (2016), Adebowale et al. (2011) and Bultosa (2007). In all three cases, our results were a little bit lower. The lower protein values found in our experiments were due to the use of another conversion factor. The 6.25 conversion factor which is used as a standard assumes that the nitrogen content of proteins is 16%. Nevertheless, the nitrogen/protein ratio is varying according to specific food products. Also important is that not all nitrogen in food is originating from proteins. Other sources of nitrogen are components like nucleic acids, urea, ammonia, nitrites, nitrates, amines, phospholipids and other (Mariotti et al., 2008). Mariotti et al. (2008), concluded that the correct conversion factor for cereals is 5.4. This conversion factor assumes that the nitrogen content in cereals is about 18.5%. Therefore, this conversion factor was used in all our calculations of the protein content of tef, resulting in a somewhat lower total protein content compared to literature. When the results found by Wrigley et al. (2016), Adebowale et al. (2011) and Bultosa (2007) were recalculated with a conversion factor of 5.4, similar results were found. In literature, all wheat results are calculated with conversion factor 6.25. For this reason, wheat was in this research calculated with conversion factor 6.25 making it easier to compare our results with literature.

The protein content of tef could be compared with the protein contents of other cereals. In the *Poaceae* family, tef is part of a totally different subfamily than the other cereals (Tatham & Shewery, 2008). In order to correctly compare the different cereals, a conversion factor of 5.4 was taken into account for all of them. The crude protein content of maize (3.89-8.53 g/100g) (Enyisi et al., 2014) and rye (7.14-7.21 g/100g) (Nilsson & Aman, 1997) were lower than the values found for tef. The content of finger millet found by Vadivoo et al. (1998) had a very broad range of crude protein content (5.79-10.71 g/100g flour) that also included the values found for tef. The protein content of barley (10.63-13.82 g/100g) (Yu et al., 2016), oat (9.50-13 g/100g) (Klose & Arendt, 2012), foxtail millet (10.99 g/100g) (Petr et al., 2003), pearl millet (10.55-11.22 g/100g) (Chowdhury & Punia, 2006) and pseudocereals like amaranth (14.95-15.51 g/100g), buckwheat (11.56-12.13 g/100g) and quinoa (12.87-13.41 g/100g) (Alvarez-Jubete et al., 2010) were higher. The results found for tef were most comparable with the crude protein content of wheat flour (7.52-10.37 g/100g) found by Kieffer et al. (1996). It should been taken into account that tef is milled into whole grain flour while wheat is not (Gebremariam et al., 2014). First of all, cereal grains also contain other sources of proteins than the endosperm like the germ and bran. In the bran, the aleurone layer contains protoplasts containing high levels of proteins (Sramkova et al., 2009). Sramkova et al. (2009), found that the protein content of wheat grain can vary between 10-18% of the total dry matter. Compared with the protein content of wheat flour, the protein content of the grains were found to be higher. Secondly, milling tef into whole grain flour can cause a dilution because of the presence of fibre in the bran and lipids in the germ (Wrigley et al., 2016). In order to make a correct comparison the grains and/or whole grain flours and/or endosperm flours of tef and wheat need to be compared.

In contrast to our results, Gebremariam et al., reviewed in 2014 that the protein content of tef was most comparable with those of barley (9.6 g/100g flour), maize (6.9-8.6 g/100g flour), pearl millet (9.8 g/100g flour) and wheat (10.1 g/100g flour). All crude protein contents calculated with conversion factor 6.25 were recalculated with a conversion factor of 5.4 in order to compare. Gebremariam et al. (2014) used the crude protein content of 9.5 g/100g of flour for tef from Bultosa and Taylor (2004). Bultosa (2007) also announced a broader range of tef crude protein content of 7.52-9.59 g/100g of flour. In this research

a tef crude protein content was found of 8.58-9.4 g/100g flour which was a little bit lower than those used by Gebremariam et al. (2014). Difference between our conclusion and those of Gebremariam et al. (2014) were because of the comparison with crude protein content in cereals found by other researchers. Different researchers have published different amounts of protein contents in cereals making it more difficult to compare. Yu et al. (2016) announced a crude protein content for barley that is 10-44% higher than those used by Gebremariam et al. (2014). For maize, Enyisi et al. (2014) found a protein content with a very broad range 3.89-8.53 g/100g of flour. This includes the range of Gebremariam et al. (2014). Both conclusions are possible in case of maize as the crude protein content of maize is only just below the crude protein content in tef. Chowdhury & Punia (2016) found a crude protein content for pearl millet that is 7-14% higher than those used by Gebremariam et al. (2014). Finally, wheat was found by Kieffer et al. (1996) to have a comparable protein content with Gebremariam et al. (2014) so this matches with our conclusions.

As the crude protein content of tef was comparable with that of wheat, it might replace wheat in human consumption without harming the nutritional value, more specific the crude protein content of the food product. If wheat is also completely replaceable by tef in food preparations more characteristics needs to be analysed. First of all, it should be characterized if wheat is replaceable by tef in order to prepare the same food products like bread, more specific the gluten content. Beside proteins other biochemical molecules are also important to analyse, as it is well known that the other macro- and micronutrients have also a functional role (Lodish et al., 2013).

In a next step of the protein characterization, the proteins were separated using the Osborn method. The amount of the protein fractions in wheat according to Kumagai (2010) were in the same order as our wheat results: glutelin (46%) > prolamin (40%) > albumin (9%) > globulin (5%). The percentages of wheat based on our average results and recovery were as followed: glutelin (45%) > prolamin (33.4%) > albumin (16.7%) > globulin (4.8%). Only the distribution of the prolamin and albumins were divergent from those found by Kumagai (2010). Prolamin and glutenin are the most abundant proteins of the wheat endosperm (Kumagai, 2010). They are not present in the seed coat layers and also not in the germ (Sramkova et al., 2009). The albumins and globulins are mainly found in the seed coats, the aleurone cells, and the germ. The concentrations in the endosperm are somewhat lower (Sramkova et al., 2009). As for wheat, only the endosperm were used to prepare flour, these gluten proteins were the most abundant ones (Kumagai, 2010).

According to Wrigley et al. (2015) and Mulugeta (1978), the protein fractions of tef showed a decreasing order of: glutelin > albumin > globulin > prolamin. Tatham et al. (1996) extracted the prolamin fraction in two steps resulting in a higher prolamin content. During the first step, they used 70% ethanol. The second step involved an extraction with 50% (v/v) aqueous propan-1-ol, 2% (v/v) acetic acid and 2% (v/v) of 2-mercaptoethanol. Extraction in this last step also dissolves the glutelin fraction because of the acetic acid. The presence of 2-mercaptoethanol reduces the disulfide bonds of the glutelin polymer resulting in structures comparable with prolamins soluble in ethanol. Both the acetic acid and the reducing agent result in no good separation of the prolamin and glutelin fractions of tef. According to Adebowale et al. (2011), the amount of protein fraction in tef is the highest for prolamin, next glutelin and at last the albumins and globulins together. Adebowale et al. (2011) showed a prolamin content of 40% of the total proteins in tef. This value is a result of an extraction performed with 60% tert-butanol containing 0.05% DTT. Also, Wrigley et al. (2016) describes results based on experiments performed with tert-butanol containing 0.05% 1,4-dithiothreitol resulting in a prolamin content of 38-43%. This is in contrast with his previous prolamin result of 3-14%. As they did not analyze the albumin, globulin

and glutelin fraction anymore it is not clear if this method results in a drop of one of these protein contents. According to Adebowale et al. (2011), a large proportion of the storage proteins in cereals are bound in a large polymeric network by disulfide bonds. For this reason, these authors presume that adding a reducing agent like DTT is necessary to extract these proteins. This method is often used to extract prolamins in tropical cereals like sorghum (Adebowale et al., 2011). It can be concluded that adding a reducing agent is less recommended because breaking the disulfide bonds results in a destruction of the glutelin network. When this happens glutelins could be extracted together with the prolamins, resulting in an overestimation of prolamins while undermining glutelins.

Adebowale et al. (2011) suggest that prolamins in tropical cereals are more hydrophobic than in wheat. For this reason, they used butanol instead of ethanol which is a more hydrophobic solvent (Adebowale et al., 2011). The hydrophobic characteristics of proteins are based on the amino acids profile. Hydrophobic amino acids are alanine, isoleucine, leucine, phenylalanine, valine, proline and glycine (Wampler, 2010). The content of the hydrophobic amino acids in g/100g of protein in tef are given as an average of two studies reported in literature (Wrigley et al., 2016; Gebremariam et al., 2014): alanine (10.1), glycine (3.1), isoleucine (4.04), leucine (8.32), phenylalanine (5.35), proline (8.2) and valine (5.68). The hydrophobic amino acids account for about 44.4 - 45.15 g/100g protein in tef. Wrigley et al. (2016) and Gebremariam et al. (2014) found that glutamic acid, alanine, proline, aspartic acid, leucine, and valine are the most abundant amino acids in tef. Four of the six previous amino acids are also hydrophobic. The content of the hydrophobic amino acids in wheat in g/100g of total protein found by Gobbetti & Ganzle (2013) are alanine (4.3), glycine (6.1), isoleucine (3.8), leucine (6.8), phenylalanine (3.8), proline (12.6) and valine (4.9). These hydrophobic amino acids account for 42.3 g/100g of total protein in wheat (Gobbetti & Ganzle, 2013). Comparing the hydrophobic amino acids of wheat with those of tef showed that the content of alanine is 2.3 times higher than those in wheat. The content of isoleucine, leucine, phenylalanine and valine are also higher in tef. The content of glycine and proline are lower in tef. There might be concluded that the hydrophobicity of tef is a little bit higher than that of wheat. The hydrophobic amino acids in sorghum are according to Mossé et al. (1988) in g/100g of total protein: alanine (9.1), glycine (3.4), isoleucine (4.1), leucine (12.7), phenylalanine (5.2), proline (8.1) and valine (5.2). The total amount of hydrophobic amino acids in sorghum account for 47.8 g/100g of total protein. Compared to wheat, sorghum is much more hydrophobic. As described by Adebowale et al. (2011) sorghum, a tropical cereal, is indeed more hydrophobic than wheat. The hydrophobicity of tef is ranging between that of sorghum and wheat. For this reason, using butanol instead of ethanol might be recommended.

Another point of attention is the low recovery of the proteins (18.3-24.5 g/100g of protein). According to the small standard deviations, there was a good replication between the different extraction batches. Wheat, used as a control, showed a much higher recovery rate. Therefore, it can be concluded that the efficiency of the Osborn method could possibly be depending on the starting material. According to Wrigley et al. (2016), tef contains individual protein bodies in the inner layer of the endosperm. In contrast, these protein bodies form a uniform matrix in wheat. This difference in anatomy between these cereals can also be an influencing factor during the Osborn fractionation. Also, tef is milled into whole grain flour resulting in the presence of potential disturbing components such as fibres, minerals, vitamins and phenolic compounds (Gebremariam et al., 2014). The bran consists of more than 50% of insoluble fibre necessary to protect the grain and endosperm (Sramkova et al., 2009). Fibre is able to form a gel around proteins and amino acids resulting in a lower availability of the proteins (Friedman, 1989). Wheat endosperm contains only 1.5% of fibre thus wheat flour proteins encounter less interference by fibres.

Interactions with lipids present in the flour can make the proteins insoluble. Lipids occur mostly in the germ but also in a lower amount in the bran and endosperm (Sramkova et al., 2009). A possible technique to remove fat is the treatment of the flours with hexane (Moroni et al., 2010). Carbohydrates, present in high amounts in the bran as well as in the endosperm are able to form complexes with proteins (Sramkova et al., 2009). To remove the carbohydrates, the flours can be treated with alfa-amylase. However, it should be taken into account that this enzyme is water soluble and so can influence the albumin and globulin contents (Adebowale et al., 2011).

Sramkova et al. (2009) declared that molecular weights of the protein fractions of wheat are the smallest for albumins and globulins. The gluten proteins, prolamins and glutelins, have the highest molecular weights. As seen on the SDS-PAGE, wheat which contains especially gliadins and glutenins have also more high molecular weight proteins. The protein pattern of the commercial gluten-free flour contains more mediate to small molecular weight proteins as they are presumed to have only very small amounts of gluten proteins. Our SDS-PAGE supported the conclusion of Sramkova et al. (2009) for wheat but not for tef. For tef, prolamins and glutelins have smaller molecular weight proteins compared with wheat. The globulins are mostly comparable in molecular weight, but the albumins showed some higher molecular weights than wheat.

The Osborn method used in this research was chosen because of its worldwide use on different cereals. As seen in the SDS-PAGE of the protein fractions of four tef varieties there was a good separation of the proteins and also the reproducibility was found to be very good. In order to advance the recovery, some changes on the protocol might be necessary.

Worldwide interest in tef has grown due to the fact that tef is believed to be a gluten-free cereal. This belief is relying on only one epidemiological study performed in The Netherlands (Hopman et al. 2008). Never before the gluten content of tef was determined. To determine the gluten content a technique was used as described in Codex Alimentarius. ELISA-results confirmed that tef indeed is a gluten-free cereal.

Some comments have to be made about the use of the commercial kit. The R5-antibodies react with specific amino acid sequences QQPFP, QLPFP, LQPFP and QQQFP in prolamins that occur in wheat, barley, and rye. The presence of glutamine and glutamic acid in or around this epitope decreases the binding with R5-antibodies (Kahlenberg et al., 2006). R-Biopharm AG (2012) declares that the test kit used during our experiments is suitable for tef flour. As there was an interaction with the R5-antibody this amino acid sequence must be present in tef flour. Cannarozzi et al. (2014) sequenced the complete genome of tef. Three of the specific amino acid sequences are found back in the genome of tef: QQPFP, LQPFP and QQQFP. To be completely sure that these peptides are related to the presence of gluten proteins, tef prolamins needs to be sequenced.

The kit presumes that prolamins account for about 50% of the gluten proteins. For wheat, this is more or less true. According to Kumagai (2010), the prolamin content in wheat is 40 g/100g of protein and the glutenin content is 46 g/100g of protein. According to our results, the glutelin content of wheat is higher (38.6 g/100g of protein) than the prolamin content (28.6 g/100g of protein). This means that the prolamin fraction accounts for only 43% of the total gluten fraction. In case of our tef results, the prolamin fraction (85%) was much higher than the glutelin fraction (15%). Eventually, the higher amount of prolamins in tef would even lower the total gluten content. So it can be concluded that if the amino acid sequencing confirms the presence of QQPFP or one of the other peptides, tef is a gluten-free cereal.

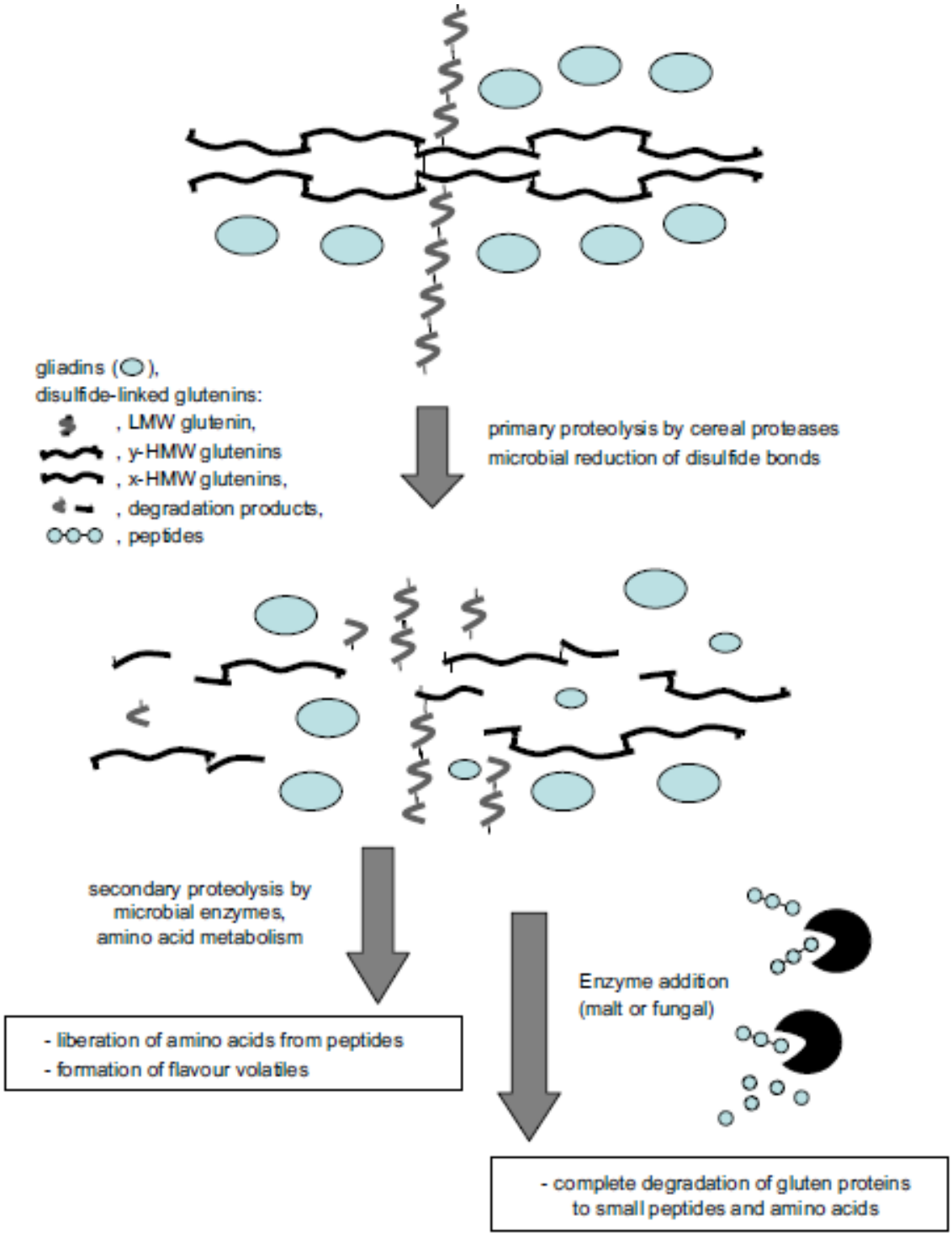
For future research, these findings might be confirmed by a western blot. Proteins separated during SDS-PAGE can be transferred onto a membrane. By the use of R5-antibody or human sera from gluten-allergic patients the gluten proteins can be immunologically detected (Mena et al., 2012). Another option is a confirmation by mass spectrometry based technologies. One of these technologies is the matrix assisted laser desorption/ionisation time-of-flight analyser mass spectrometry (MALDI-TOF-MS) for the detection of intact gluten proteins. These novel techniques are used for quantitative gluten analyses (Colgrave et al., 2014).

This study reports IVPD of tef flour and tef food products for the first. The IVPD results from tef flour could be compared with the results of other cereals. The IVPD of tef flour were more digestible than those of barley (Sher et al., 2011), finger millet (Antony & Chandra, 1998), maize (Duodu et al., 2012) and oat (Li & Xu, 2015). The digestibility of tef proteins was comparable with those of buckwheat (Dogra & Awasthi, 2015) and pearl millet (Chowdhury & Punia, 2006). On the other hand, the IVPD of amaranth (Bejosano & Corke, 1998), quinoa (Elohaimy et al., 2015), rye (Aura et al., 1999) and wheat (Abdel-Aal & Hucle, 2002) were higher than those of the tef varieties. It should be noted that not all IVPDs found in the literature were performed in the same way, with the same enzymes or with the same starting material. These results mean that the conclusions of Wrigley et al. (2016), who declared that IVPD of tef is presumed to be higher than in other cereals, were not correct. The conclusions of Wrigley et al. (2016) are based on the fact that they believe that albumins and globulins are more digestible. In contradiction, wheat was found to contain higher amounts of prolamins and glutenins but their IVPD is even higher than that of tef. Friedman (1989), declared that proteins like albumins and globulins which are more soluble are also more digestible. As both, protein solubility and protein digestibility, are strongly influenced by other components in the cereals, this conclusion is not completely guaranteed. Proteins are able to conjugate with carbohydrates, lipids, nucleic acids and metals resulting in a lower digestibility. Another influencing factor is that not all peptide bonds are available for cleavage (Friedman, 1989). The presence of tannins is also a factor resulting in the decrease of protein digestibility due to their characteristic to bind with proteins (Friedman, 1989; Dykes & Rooney, 2007). Recent research performed by Shumoy et al. (2017) on the same seven tef varieties as used in this research showed tannin contents ranging from 65-302 mg catechin equivalent/100g dry matter flour. So it could be concluded that tannins might play a role in the IVPD. Friedman (1989) also described that the presence of fibre inhibits the availability of proteins for cleavage.

The method used in these experiments was based on a pH-drop. All three enzymes used in this experiment cut at the C-terminal side of the amino acids (Sigma-Aldrich, 2017a; Sigma-Aldrich, 2017b, Sigma-Aldrich, 2017c). Cleavage of the proteins by these enzymes at alkaline pH, leads to the release of peptides, amino acids and more importantly to the release of protons resulting in a drop of pH (Moyano et al., 2015; Tinus et al., 2012). Another point of attention is the formula used to calculate the IVPD. First, this equilibrium will even if no digestion occurs, give 66% IVPD. Second, percentages above 100 are possible if the change in pH after 10 minutes is ≥ 1.9 (Tinus et al., 2012). There were results of other cereals found with IVPDs below 66%. These results are found with another technique in which they calculate the IVPD according to the nitrogen content before and after the in vitro protein digestion. The pH-drop method can and already was criticized before because of its simplicity compared with the complex processes taking place in vivo. Also, food components with buffering capacity can influence the pH-drop. A lot of techniques, from very simple to very complex, are now available to determine the protein digestibility (Moyano et al., 2015). From the simple techniques, the pH-drop of Hsu et al. (1977) and the pH-stat assays of Pedersen & Eggum (1983) are most frequently used. More

complex methods are invented to completely simulate hydrolysis and intestinal absorption of nutrients (Moyano et al., 2015). Although the pH-drop method is criticized by some researches this method was chosen because it still is the most used technique worldwide due to its simplicity and the relatively low cost.

Results from the SDS-PAGE of the fermented dough and *injera* showed that the main difference in the protein pattern took place during the fermentation process. During fermentation two main steps of proteolysis takes place (Fig. 5.1). Proteases are divided into proteinases and peptidases. The first group is able to degrade proteins into smaller peptide fractions. The peptidases hydrolyze peptides into amino acids. During the primary proteolysis, lactic acid bacteria produce lactic acid resulting in a drop of pH to 3.5-4. Cereal aspartic proteinases perform at this pH a primary proteolysis. Lactic acid bacteria also have a limited proteolytic activity. Drop in pH and reduction of disulfide bonds in gluten proteins results in an increased solubility and susceptibility for degradation of the gluten proteins. During a second proteolysis, lactic acid bacteria hydrolyzes peptides into amino acids. The release of amino acids and peptides are influencing taste and flavor of the final product. The increase of amino acids can also be compensated by yeast. As there was a natural fermentation performed in this research, yeast could influence the fermentation. The presence of malt and fungal proteases results in reduced gluten contents suitable for people with gluten sensitivities. Gluten proteins are important factors in determining physical properties of food products (Gänzle et al., 2008; Poutanen et al., 2009).



Figuur 5.1: Proteolytic pathway during fermentation (Gänzle et al., 2008)

Baking only showed a small influence on protein concentration but not in protein pattern. Heat makes the proteins undergo denaturation forcing proteins into the primary structure. No peptide bonds are broken during this process (Lodish et al., 2013). During bread making, gluten proteins are necessary for the development of the physical properties of the final product. Yeast produces carbon dioxide during fermentation. The gluten network traps this gas, resulting in changing of the dough into a light and open texture (The National Academies, 1996). During the heating step, gluten proteins undergo structural changes as a result of increased SH- and SS-interchange reaction. These changes result in an increase of cross-linking and polymerization necessary during bread making (Sivam et al., 2010). Low prolamin and glutelin concentration found in tef have its influence on the physical properties of *injera*. For this reason and also its high moisture content, *injera* distinguishes itself from other bread because of its chewy and elastic properties (The National Academies, 1996).

Finally, possible allergens of tef flour, fermented dough and *injera* were given based on their molecular weight. It needs to be stressed that SDS-PAGE only gives an estimation of the molecular weight and so these are only possible allergens of tef. The study of allergens in food products is necessary in order to label correctly and to protect the consumers. Nevertheless, detection of allergens is not always easy because they can occur in low concentrations or they can be masked by the food matrix. Methods to detect allergens are based on protein or DNA detection. In order to guaranty the presence of allergens further research is necessary. For the detection of specific allergens, human IgE blotting can be performed after SDS-PAGE. Disadvantages of this method are the time-taking methods and a lack of appropriate human sera. If allergens are characterized the human sera can be replaced by antibodies produced in animals (Poms et al., 2004).

Many of the allergens already detected in different cereals could also possibly be present in tef. In total, 42 allergens could possibly occur in tef, while in wheat there were 51 found. The techniques used in this research did not guaranty the presence of these allergens in tef. For this reason, they were all marked as possible allergens. A lot of these allergens were only yet detected in one single type of cereal, mostly wheat. The allergens found in multiple types of cereals also have more chance to occur in tef. Allergens that possibly occur in tef are part of the cupin superfamily, prolamin superfamily, plant defense system and enzymes. After the fermentation step, a lot of these allergens were disappeared. Only 17 of the allergens that possibly occurred in tef flour were found back after the food preparation. As tef is almost never consumed as a raw product but as a food preparation, this result is very interesting. According to M'hir et al. (2012) and Verhoeckx et al. (2015), gluten and also other allergens are able to undergo hydrolysis during fermentation. Degradation is first of all possible because of the presence of germinating cereal proteases. These are endogenous proteases synthesized during cereal germination. Also bacterial and/or fungal enzymes present during fermentation are able to break down gluten (M'hir et al., 2012). Verhoeckx et al. (2015) claims that fermentation is a good method to reduce the amount of allergens in food products. For this reason, the present enzymes during fermentation get a lot of attention now-a-days as manipulation of food products in order to reduce the gluten content. The influence of heat on gluten depends on the type of protein and the heating procedure. In most cases, the amount of allergic epitopes are reduced by heating (Verhoeckx et al., 2015; Shin et al., 2012).

6 Conclusion

In the last decades interest has grown in new types of cereals to replace gluten-containing cereals. *Eragrostis tef* as one of these cereals, is gaining wide acceptance in the global market.

A protein characterization of tef cereal was performed. The crude protein content of seven different tef varieties was found to be between 8.48 and 9.40 g/100g of flour (db) and are more or less similar to other cereal grains. The content of the globulins was the highest in tef, followed by the albumins, prolamins and glutelins. A good separation of the Osborn fractions was found in each fraction of different tef varieties as seen by SDS-PAGE. Further optimization of the Osborn fractionation with tef as starting material might be necessary as the recovery rate was very low. In all the seven tef varieties the gluten content was found to be below 20 mg/kg. According to the European Commission tef may now be labelled as a gluten-free food product. The IVPD of tef flour was found to be 71.5% and no significance difference was found among the seven tef varieties. Also IVPD was tested on *injera* for which the results were slightly higher in case of *injera* compared to the flour. Protein pattern of the SDS-PAGE showed that fermentation could change the molecular size pattern of tef proteins. Finally, the possible presence of allergens in tef was tested by SDS-PAGE. Possible allergens found in tef are part of the cupin superfamily, prolamin superfamily, plant defense system and enzymes. Further research is necessary to confirm the presence of these allergens in tef. The number of allergens are probably reduced after a fermentation step by the presence of cereal proteases, bacterial and/or fungal enzymes.

Based on all of these results there could be concluded that nutritionally, tef might be a good replacement for gluten-containing cereals like wheat. Especially important for people with gluten allergies, gluten intolerance or celiac disease, tef was found to be gluten-free. This information is also really important for later research on wheat replacement by tef in food preparations like bread. Nevertheless, further research on other biochemical molecules of tef are necessary when tef wants to be implemented as a replacement for wheat. In general, tef seems to be a promising cereal.

7 References

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Appendix 1: Allergens in different cereals, their molecular weight and routes of exposure

(WHO/IUIS Allergen Nomenclature Sub-Committee, n.d; Allergome, 2015)

	Allergen	MW (kDa)	Tissues	Routes of exposure
Amaranth	Ama r 1: Ole e 1-like protein (Trypsin inhibitor)	18	Pollen	Inhalation
	Ama r 2/Ama v 2: Profilin (Actin binding protein)	14	Pollen	Inhalation
Barley	Hor v 1: Grasses group 1: Expansin	28	Pollen	Inhalation
	Hor v 2: Grasses group 2	12	Pollen	Inhalation
	Hor v 4: Grasses group 4: Berberine bridge enzyme	60	Pollen	Inhalation
	Hor v 5: Grasses group 5: Ribonuclease	30	Pollen	Inhalation
	Hor v 7: Polcalcin (Calcium-binding protein)	9	Pollen	Inhalation
	Hor v 7k-LTP	12	Seed	Ingestion
	Hor v 12: Profilin (Actin binding protein)	14.3	Pollen	Inhalation
	Hor v 13: Polygalacturonase	46	Pollen	Inhalation
	Hor v 14: Lipid-transfer protein	9	Seed	Ingestion
	Hor v 15: Alpha-amylase inhibitor BMAI-1 precursor	16	Seed	Ingestion/ Inhalation
	Hor v 16: Alpha-amylase	47.8	Seed	Ingestion
	Hor v 17: Bèta-amylase	57.3	Seed	Ingestion
	Hor v 18kD	18	Seed	Ingestion
	Hor v 20: Gamma-hordein 3	33.1	Seed	Ingestion
	Hor v 28: Alpha-amylase inhibitor	15	Seed	Ingestion
	Hor v 32: Peroxiredoxine	24	Seed	Ingestion
	Hor v 33: Serpin (Trypsin inhibitor)	45	Seed	Ingestion
	Hor v 36: Glutenin	35	Seed	Ingestion
	Hor v 37: Purothionin	15	Seed	Ingestion
	Hor v 39: Serine protease inhibitor	9	Seed	Inhalation
	Hor v BDAI: alpha-amylase inhibitor	16.43	Seed	Inhalation
	Hor v BTI: Trypsin inhibitor	16.14	Seed	Inhalation
	Hor v GBSS_I: Starch synthase	66	Seed	Ingestion
Buckwheat	Fag e 1/Fag t 1: 13S/11S Globulin	24	Seed	Ingestion
	Fag e 2/ Fag t 2: 2S albumin	16	Seed	Ingestion
	Fag e 3: 7S Vicilin	19	Seed	Ingestion
	Fag e 10kD/ Fag t 10kD: Alpha-amylase/trypsin inhibitor	10	Seed	Ingestion
	Fag e TI: Trypsin inhibitor	7	Seed	Ingestion
Millet	Pan mi GBSS_I: Starch synthase	58	Seed	Ingestion
	Set it 1: Grasses group 1: Expansin	29	Pollen	Inhalation
	Set it 11: Ole e 1-like (Trypsin inhibitor)	18	Pollen	Inhalation
	Set it 12: Profilin (Actin-binding protein)	21	Pollen	Inhalation
	Set it 13: Grasses group 13: Polygalacturonase	43	Pollen	Inhalation
	Set it GBSS_I: Starch synthase	66	Seed	Ingestion/ Inhalation

	Allergen	MW (kDa)	Tissues	Routes of exposure
Maize	Zea m 1: Grasses group 1: Bèta-expansin	35	Pollen	Inhalation
	Zea m 2: Grasses group 2	32	Pollen	Inhalation
	Zea m 3: Grasses group 3	13	Pollen	Inhalation
	Zea m 4: Grasses group 4: Berberine bridge enzyme	60	Pollen	Inhalation
	Zea m 5: Grasses group 5: Ribonuclease	30	Pollen	Inhalation
	Zea m 7: Polcalcin (Calcium-binding protein)	9	Pollen	Inhalation
	Zea m 8: Chitinase	28.98	Seed	Ingestion
	Zea m 11: Ole e 1-like protein (Trypsin inhibitor)	16	Pollen/ Seed	Inhalation
	Zea m 12: Profilin (Actin-binding protein)	14	Pollen	Inhalation
	Zea m 13: Grasses group 13: Polygalacturonase	60	Pollen	Inhalation
	Zea m 14: 9k-LTP	9	Seed	Ingestion
	Zea m 20S: Proteasome subunit	23.07	Seed	Ingestion
	Zea m 22: Enolase (Glycolytic enzyme)	48.2	Pollen	Inhalation
	Zea m 25: Thioredoxin	14	Seed	Ingestion
	Zea m 27: Glutenin subunit	27	Seed	Ingestion
	Zea m 32: Peroxiredoxine	23	Seed	Ingestion
	Zea m 50: Glutenin subunit	34.6	Seed	Ingestion
	Zea m G1: 7S Vicilin	66.16	Seed	Ingestion
	Zea m G2: Globulin-like protein	49.89	Seed	Ingestion
	Zea m GBSS_I: Starch synthase	66	Seed	Ingestion
	Zea m PAO: Amino oxidase	56.3	Leaf	/
	Zea m Zm13: Ole e 1-like (Tryspin inhibitor)	18	Pollen	Inhalation
Oats	Ave s 1: Grasses group 1: Expansin	33	Pollen	Inhalation
	Ave s 2: Grasses group 2	12	Pollen	Inhalation
	Ave s 4: Grasses group 4: Berberine bridge enzyme	60	Pollen	Inhalation
	Ave s 5: Grasses group 5: Ribonuclease	29	Pollen	Inhalation
	Ave s 7: Polcalcin (Calcium-binding protein)	/	Pollen	Inhalation
	Ave s 11S: 11S Globulin	23	Seed	Ingestion
	Ave s 12: Profilin (Actin-binding protein)	14	Pollen	Inhalation
	Ave s 13: Grasses group 13: Polygalacturonase	/	Pollen	Inhalation
	Ave s 36: Glutenin	25	Seed	Ingestion
	Tri p 1: Grasses group 1: Expansin	/	Pollen	Inhalation
	Tri p 5: Grasses group 5: Ribonuclease	/	Pollen	Inhalation

	Allergen	MW (kDa)	Tissues	Routes of exposure
Rye	Sec c 1/Lol p 1: Grasses group 1: Expansin	30	Pollen	Inhalation
	Sec c 2: Grasses group 2	12	Pollen	Inhalation
	Sec c 3: Grasses group 3	/	Pollen	Inhalation
	Sec c 4: Grasses group 4: Berberine bridge enzyme	60	Pollen	Inhalation
	Sec c 5: Grasses group 5: Ribonuclease	35	Pollen	Inhalation
	Sec c 12: Profilin (Actin-binding protein)	14	Pollen/ seed	Inhalation
	Sec c 13: Grasses group 13: Polygalacturonase	/	Pollen	Inhalation
	Sec c 20: Prolamin	70	Seed	Ingestion
	Sec c 28: Alpha-amylase inhibitor	15	Seed	Ingestion
	Sec c 32: Peroxiredoxine	23	Seed	Ingestion
	Sec c 36: Glutenin	70	Seed	Ingestion/ Inhalation
	Sec c 37: Purothionin	15	Seed	Ingestion
	Sec c 38: Dimeric alpha-amylase/trypsin inhibitor	13.5	Seed	Ingestion/ Inhalation
	Sec c GBSS_I: Starch synthase	/	Seed	Ingestion
	Lol p 2: Grasses group 2	10	Pollen	Inhalation
	Lol p 3: Grasses group 3	10	Pollen	Inhalation
	Lol p 4: Grasses group 4: Berberine bridge enzyme	60	Pollen	Inhalation
	Lol p 5: Grasses group 5: Ribonuclease	35	Pollen	Inhalation
	Lol p 7: Polcalcin (Calcium-binding protein)	9	Pollen	Inhalation
	Lol p 10: Cytochrome C	12	Pollen	Inhalation
	Lol p 11: Ole e 1-like protein (Trypsin inhibitor)	14.8	Pollen	Inhalation
	Lol p 12: Profilin (Actin-binding protein)	14	Pollen	Inhalation
	Lol p 13: Grasses group 13: Polygalacturonase	60	Pollen	Inhalation
	Lol p Cyp: Cyclophilin (Rotamase)	26	Pollen	Inhalation
	Lol p FT: Fructosyltransferase (Hydrolase)	71.3	Pollen	Inhalation
	Lol p Legumin: 11S Globulin (Legumin-like protein)	38	Pollen	Inhalation

	Allergen	MW (kDa)	Tissues	Routes of exposure
Wheat	Tri a 1: Grasses group 1: Expansin	29.97	Pollen	Inhalation
	Tri a 2: Grasses group 2	12	Pollen	Inhalation
	Tri a 3: Grasses group 3	13	Pollen	Inhalation
	Tri a 4: Grasses group 4: Berberine bridge enzyme	60	Pollen	Inhalation
	Tri a 5: Grasses group 5: Ribonuclease	/	Pollen	Inhalation
	Tri a 7: Polcalcin (Calcium-binding protein)	/	Pollen	Inhalation
	Tri a 7k-LTP	7	Seed	Ingestion/ Inhalation
	Tri a 10kD	10	Seed	Ingestion
	Tri a 12: Profilin (Actin-binding protein)	14	Pollen/ seed	Ingestion/ Inhalation
	Tri a 13: Grasses group 13: Polygalacturonase	/	Pollen	Inhalation
	Tri a 14: 9k-LTP	9	Seed	Ingestion/ Inhalation
	Tri a 15: Monomeric alpha-amylase inhibitor 0.28	15	Seed	Inhalation
	Tri a 18: Agglutinin isolectin 1	21.24	Seed	Ingestion
	Tri a 19: Omega-5 gliadin (Seed storage protein)	65	Seed	Ingestion
	Tri a 20: Gamma-gliadin	32.6	Seed	Ingestion
	Tri a 21: Alpha-bèta-gliadin	33	Seed	Inhalation
	Tri a 23kd: Leucine-rich repeat protein	15	Seed	Ingestion
	Tri a 25: Thioredoxin	13	Seed	Ingestion
	Tri a 26: HMW glutenin	88	Seed	Ingestion
	Tri a 27: Thiol reductase homologue	27	Seed	Inhalation
	Tri a 28: Alpha-amylase inhibitor 0.19	13.34	Seed	Inhalation
	Tri a 29: Alpha-amylase inhibitor CM2	15.4	Seed	Inhalation
	Tri a 30: Alpha-amylase inhibitor CM3	16	Seed	Ingestion/ Inhalation
	Tri a 31: Triosephosphate-isomerase	27	Seed	Inhalation
	Tri a 32: 1-cys-peroxiredoxin	23	Seed	Inhalation
	Tri a 33: Serpin (Trypsin inhibitor)	43	Seed	Ingestion/ Inhalation
	Tri a 34: Glyceraldehyde-3-phosphate-dehydrogenase	37	Seed	Inhalation
	Tri a 35: Dehydrin	12	Seed	Inhalation
	Tri a 36: LMW glutenin GluB3-23	46	Seed	Ingestion
	Tri a 37: Alpha-purothionin	12	Seed	Ingestion
	Tri a 39: Serine protease inhibitor	9	Seed	Inhalation
	Tri a 40: Alpha-amylase/trypsin inhibitor	15.78	Seed	Inhalation
	Tri a 41: Mitochondrial ubiquitin ligase activator of NFKB 1-like protein	20.25	Seed	Ingestion
	Tri a 42: Grasses group 42	31.46	Seed	Ingestion
	Tri a 43: Grasses group 43	35.64	Seed	Ingestion
	Tri a 44: Endosperm transfer cell-specific PR60 (Endosper transfer cell specific protein)	35.53	Seed	Ingestion
	Tri a 45: Elongation factor 1 (EIF1)	33.55	Seed	Ingestion

	Allergen	MW (kDa)	Tissues	Routes of exposure
Wheat	Tri a aA_SI: Alpha-amylase/subtilisin inhibitor	19.6	Seed	Ingestion
	Tri a alpha_gliadin	33	Seed	Ingestion
	Tri a bA: Bèta-amylase (gluten)	60	Seed	Ingestion
	Tri a Bd36K: Peroxidase	36	Seed	Inhalation
	Tri a beta_gliadin (gluten)	32.9	Seed	Ingestion
	Tri a Chitinase	30	Seed	Ingestion
	Tri a CMX: Alpha-amylase/trypsin inhibitor	13.9	Seed	Ingestion
	Tri a DH: dehydrin	33.4	Seed	Inhalation
	Tri a Endochitinase	67	Seed	Ingestion
	Tri a GBSS_I: Starch synthase	/	Seed	Ingestion
	Tri a Germin: Germin-like protein	65	Seed	Inhalation
	Tri a Gliadin (gluten)	23	Seed	Ingestion
	Tri a GST (Glutathione-S-transferase)	/	Seed	Inhalation
	Tri a LMW-GS B16: Glutenin subunit	/	Seed	Ingestion
	Tri a LMW-GS P42: Glutenin subunit	/	Seed	Ingestion
	Tri a LMW-GS P73: Glutenin subunit	30	Seed	Ingestion
	Tri a omega2_Gliadin (gluten)	37.96	Seed	Ingestion
	Tri a PAP: Purple acid phosphatase	60	Seed	Contact skin
	Tri a Peroxidase	38.8	Seed	Ingestion
	Tri a Peroxidase 1	23	Seed	Ingestion
	Tri a TLP: Thaumatin-like protein	29.6	Seed	Ingestion
	Tri a Tritin: Ribosomal inactivating protein	13	Seed	Ingestion
	Tri a Trx: Thioredoxin (oxidoreductase)	33.2	Seed	Inhalation
	Tri a XI: Xylanase inhibitor	15	Seed	Ingestion
	Tri m 37: Purothionin	66	Seed	Ingestion
	Tri m GBSS_I: Starch synthase	36	Seed	Ingestion
	Tri m Peroxidase	9	Seed	Inhalation
	Tri td 14: LTP	14	Seed	Ingestion
	Tri td 28: Alpha-amylase inhibitor	16	Seed	Ingestion
	Tri td 30: Alpha-amylase/trypsin inhibitor	10	Seed	Ingestion
	Tri td 7k-LTP	66	Seed	Ingestion
	Tri td GBSS_I: Starch synthase	85-91	Seed	Ingestion
	Tri tp 26: Glutenin subunit	22	Seed	Ingestion
	Tri ur 1: Grasses group 1: CCD-bearing protein, expansin	10	Pollen	Inhalation
	Tri ur 2: Grasses group 2	13	Pollen	Inhalation
	Tri ur 3: Grasses group 3	30	Pollen	Inhalation
	Tri ur 5: Grasses group 5: Ribonuclease	79-90	Pollen	Inhalation
	Tri ur 26: Glutenin subunit	14	Seed	Ingestion
	Tri ur 28: Alpha-amylase inhibitor	15	Seed	Ingestion
	Tri ur 37: Purothionin		Seed	Ingestion

Appendix 2: Protease, trypsin and chymotrypsin

	Protease	Trypsin	Chymotrypsin
Optimum pH	5.0-9.0	7.0-9.0	7.8
Place of cutting	Hydrolyze peptide bonds on the carboxyl side of glutamic or aspartic acid	Cleaves peptides on the C-terminal side of lysine and arginine residues.	Hydrolyzes peptide bonds with aromatic or large hydrophobic side chains (Tyr, Trp, Phe, Met, Leu) on the carboxyl-end of the bond.
Unit	One unit will hydrolyze casein to produce color equivalent to 1.0 μmole (181 μg) of tyrosine per min at pH 7.5 at 37 °C (color by Folin-Ciocalteu reagent).	One BAEE unit will produce a A253 of 0.001 per minute at pH 7.6 at 25°C using BAEE as a substrate.	One unit will hydrolyze 1.0 μmole of BTEE per min at pH 7.8 at 25 °C.