Determining the Impact of Genotype × Environment on Oat Protein Isolate Structure, Function, and Composition

by

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Abstract

Oat has recently received widespread attention owing to its relatively high protein content (15-20%) among cereals. Given the increased use of plant proteins in the food industry, oat protein isolate is also considered a sustainable plant protein alternative. The characteristics of oat protein depend on both genotype and the environment in which it is grown. Therefore, the genotype × environment interaction (G × E) has a major influence on protein composition, structure, and functionality which is of great importance in the context of end product quality and consistency. The goal of the first study of the thesis was to evaluate the effect of G × E on oat protein structural characteristics and functionality using three oat genotypes (Summit, AC Morgan, and CS Camden) grown in three different environments (Manitoba, Alberta, and Saskatchewan) in the Canadian Prairies. The second study aimed at determining the effect of G × E on the relative protein composition through SE-HPLC and the use of LC-MS for protein identification. Oat protein isolate (OPI) was extracted from defatted oat flour at pH 9 at a 1:5 flour:NaOH ratio and precipitated at pH 5.3. The OPI samples were freeze-dried and evaluated for structural characteristics such as protein profile, surface hydrophobicity, secondary structure, and denaturation characteristics, as well as functional properties such as solubility, foaming, emulsification, and gelling. All structural and functional tests were conducted at pH 7. The first study indicated that oat protein content, protein profile, and functional properties are dependent on genotype and the environment. The surface hydrophobicity indicated a stronger impact from the growing environment and samples from Alberta showed the highest surface hydrophobicity. The denaturation temperature for OPI ranged from 110.2 -111.6 °C. The water solubility of OPI was significantly impacted by G × E, where the solubility ranged from 13-30 %. Samples Alberta – Summit exhibited the highest

foaming capacity, while all samples tested had good foaming stability >70%. Furthermore, it was found that G×E significantly impacts OPI structure and functionality including denaturation enthalpy, protein solubility, foaming capacity, emulsion stability, and textural characteristics. The SE-HPLC separated the OPI into four major fractions including polymeric globulin, avenins, glutelins and albumins as well as smaller peptides, whereas LC-MS was able to identify eight major types of proteins present in OPI including globulins, prolamins/avenins, glutelins, enzymes/ albumins, enzyme inhibitors, heat shock proteins, grain softness proteins and allergenic proteins. The SE-HPLC analysis revealed that environment has a stronger effect on overall oat protein composition, while LC-MS results indicated a significant genotypic effect on the main globulin protein type in OPI. PCA results indicated that certain environment and genotypic combinations could be better choices in terms of globulin protein quality given their positive and negative associations with certain genotypes and environments. Overall, the results indicate that genotype, environment and G×E significantly impact OPI structure, functionality and composition, highlighting the need for future work to further expand the examination of OPI structure-function and compositional characteristics to determine oat cultivars that can be used to produce OPI of consistent quality. To that end, this study provides new insights into the selection of oat genotypes and environment combinations for targeted protein applications in the food industry.

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Dedication

This thesis is dedicated

To my beloved father and mother,

Rupes Mel and Swarna Herath

for their endless love and unimaginable dedication to making me who I am today…

to my husband, Mihiran

for his love and patience with me…

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List of Abbreviations

AE-IEP - Alkaline extraction-isoelectric precipitation

OPI – Oat protein isolate

OPC – Oat protein concentrate

G×E - Genotype × Environment interaction

G and Es - Genotypes and Environments

SE-HPLC – Size exclusion- High performance liquid chromatography

LC-MS – Liquid Chromatography – Mass Spectrometry

CAGR – Compound annual growth rate

SC-CO_2 – Super critical carbon dioxide

BSA – Bovine serum albumin

DES – Deep Eutectic solvents

EAA – Essential amino acids

WHC – Water hydration capacity

SDS -PAGE – Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

FTIR – Fourier Transform Infrared spectroscopy

GMO – Genetically modified organisms

ANOVA – Analysis of variance

PCA – Principal Component Analysis

Chapter 1: Introduction

Oat (*Avena sativa*) is one of the most economically important cereals in Canada and is also important from a nutritional perspective. Oat contains a significant amount of soluble dietary fiber (β-glucans), vitamin E, and polyunsaturated fatty acids apart from being a source of starch (Sterna et al., 2016). β-glucans in oat have demonstrated hypocholesterolemic and anti-cancer properties contributing to preventing heart diseases and diabetes (Sterna et al., 2016). On the other hand, oat is considered one of the highest protein-containing cereals (Boyer et al., 1992). Oat contains salt-soluble globulins as its major storage protein accounting for 60-80% of total grain protein, as opposed to wheat which has prolamins and glutelins as major storage proteins (Mark A Shotwell et al., 1988).

In the 20th century, oat production accounted for only 1% of cereal production worldwide, however due to increasing awareness of the health benefits of oat, the food uses of oat have increased to higher level in 21st century and is currently the sixth highest consumed cereal worldwide (Mäkinen et al., 2016a). Canada is the second largest oat producer (2.8 million tons) after Russia (3.8 million tons) and it is also grown in Australia, Poland, Spain, United States, Finland, Ukraine, Argentina, and Turkey (FAO STAT, 2021). Furthermore, oat is robust and resistant to common plant diseases compared to wheat or barley, and can outcompete most weeds (Mäkinen et al., 2016a). Therefore, oat is considered to be a sustainable crop when compared to other cereals such as wheat, rye, barley or corn.

Oat contains approximately12-20% protein, however, this is subject to change across genotypes and environments. The unique protein composition of oat has made it a good candidate to be used in plant-based protein products that are hypoallergenic compared to wheat gluten or soy proteins. In fact, the food industry is seeking hypoallergenic and non-GMO alternates to soy protein for

plant-based protein ingredients and meat analog production. According to Chen (2023), oat and beans have been combined to make a fiber rich meat analog, resembling pulled pork due to excellent gelling properties of oat protein. Oat has long been extracted through alkaline extraction-isoelectric precipitation (AE–IEP) methods to produce oat protein isolate (OPI) with purity >80% protein or oat protein concentrate (OPC) with relatively lower protein content of 65-80% (Ma, 1983). In this method, the proteins are first solubilized in alkaline conditions followed by their precipitation in acidic conditions at their isoelectric point.

Oat protein has long been studied for its functional properties and is recognized as a good gelling, emulsification and foaming agent, even though its solubility at neutral pH is quite less than that of animal proteins (Ma, 1983). Although native oat proteins exhibit weak gelling capacity, studies show that enzymatic modification can be used to improve their gel strength, which enhances utilization in food applications (Mäkinen et al., 2016a). Due to its good emulsifying properties, oat proteins have been used to formulate food products including heat resistant chocolates to stabilize the structure during heating (Rasane et al., 2015). Furthermore, in terms of organoleptic attributes oat protein ingredients are recognized to have better sensorial properties when compared to proteins derived from oilseeds and legumes (Boukid, 2021). Therefore, considering the hypoallergenic nature, better functionality and organoleptic properties, oat protein may have the ability to address the needs of the plant protein industry as a novel functional ingredient.

The physicochemical properties and associated functional attributes of OPI or OPC are impacted by G × E, as well as processing conditions. Consistent product quality is one of the key considerations in the food industry when choosing a new protein ingredient for product development. Even though oat protein has long been evaluated for structural and functional properties, these is a research gap regarding the impact of genotype x environment on oat protein

structure-functionality (Ma & Harwalkar, 1984). Previous studies have focused on evaluating the G × E on oat grain morphology and proximate composition, where results have indicated that oat grain characteristics such as groat percentage, grain weight and protein content are impacted by both genotype and environment (Doehlert et al., 2001). However, the G × E on oat protein structural and functional properties have not been studied in any previous work even though it is of great importance to the food industry in order to establish oat protein as a mainstream plant-protein ingredient.

Therefore, the first part of the study is based on the hypothesis that oat protein structural and functional properties are significantly impacted by G × E. The objectives of the study were to evaluate the impact of G × E on oat protein structural and functional properties using three oat genotypes (Summit, AC Morgan and CS Camden) from three environments (Manitoba, Saskatchewan and Alberta) in the Canadian prairies. The changes in oat protein structural and functional properties can be related to the changes in oat protein physicochemical properties and composition, including the distribution of different oat protein fractions. Furthermore, identification of specific peptides related with specific functionalities can contribute to the targeted development of oat protein cultivars for protein ingredient development. Additionally, a recent study suggested that oat protein techno-functional properties including foaming and emulsifying properties are directly influenced by the ratio of polymeric to monomeric proteins in protein ingredients, further enhancing the need to understand oat protein chemistry, functionality and the impact of G × E on structure and functionality (Gell et al., 2022). In this context, the second part of the study is based on the hypothesis that oat protein composition is significantly impacted by G × E. The objectives of this study were to evaluate the effect of G × E on the protein composition through SE-HPLC and use of LC-MS for further in-depth quantitation of globulin proteins present

in OPI and to determine the associations of genotypes and environments with different globulin proteins.

Chapter 2: Literature Review

Oat protein as a novel protein ingredient: structure, functionality and factors impacting utilization

A version of this chapter (sections 2.1 – 2.8) has been published in Cereal Chemistry:

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2.1. Abstract

This review outlines the current state of oat usage and potential use as a protein ingredient, oat protein extraction techniques, oat protein structure and functionality, and the interaction with genotype and environment (G × E) on oat protein quality. Oat protein has a structural similarity to soy glycinin and has the potential to be used as novel functional ingredient in food processing. The globulin protein, which accounts for about 70-80% of the oat protein content exhibits higher heat stability than most plant proteins. The protein content of oat is strongly dependent on both genotype and the environment as well as agricultural practices, such as fertilizer use. The versatility of oat protein as a food ingredient is yet to be discovered, and current research addresses some of aspects of oat protein utilization, although more research is needed to determine G × E impacts on oat protein structure and functionality. This review provides novel insights into how oat protein can be used as a functional protein ingredient in food applications and how structural properties and functionality could be influenced by G × E factors.

Keywords: oat protein, extraction, protein structure and functionality, oat globulin

2.2. Introduction

Oat has been considered as an important crop since ancient times, owing to its nutritional value (Martínez-Villaluenga & Peñas, 2017). Oat is popular for its hypocholesterolemic effects, glycemic control, and positive impact on gut microbiota, while also having exceptionally higher protein content than most other cereals. With the growing demand for sustainable protein sources, numerous studies have been conducted to review the environmental impact of the production of traditional protein sources and the need to explore more plant-based protein sources (Carlsson-Kanyama & González, 2009). It is well known that soy protein dominates the plant protein market and is being utilized widely owing to its functional properties (Singh et al., 2008). The market for alternative protein sources is expected to grow at 14% annually by 2024, while the world wide plant-based protein market is predicted to reach USD 10.8 billion by 2022 (National Research Council Canada, 2019). In 2019 the total production of soybean reached 96,793 tons, which is indicative of its high utilization (FAO STAT, 2019). Even though soy protein has long been recognized and utilized as a protein source from plant origin, it is one of the Big 8 allergens. In fact, soy, pea and tree nuts contain allergenic seed storage proteins of the Cupin superfamily (Breiteneder & Radauer, 2004). Therefore, there is a need to explore plant protein sources that are non-allergenic or hypo allergenic. However is it stated that oat protein as an emerging protein source, could replace soy proteins in non GMO food applications due to its similarity to that of dehulled soybean meal in terms of protein quality (Hahn et al., 1990). Furthermore, most cereal prolamin proteins are known for their allergenicity due to high contents of proline and glutamine; however, oat is an exception from the category as the major storage protein in oat is globulin (Shewry et al., 2002).

Oat β-glucans have been commonly studied regarding physical properties as well as nutritional attributes, specifically its ability to reduce serum cholesterol. However, there is limited literature on oat protein extraction, characterization, and applications. Protein is one of the largest component (12- 20%) followed by starch in terms of grain composition (Nieto-Nieto et al., 2015). However, the protein content varies with the climate and geographic location. Protein quality of cereal grains are determined by their amino acid composition and digestibility. With regard to essential amino acid content, oat contain high amount of lysine, asparagine-aspartic acid, and alanine which makes it superior in comparison to other cereal protein sources (Zhong et al., 2019). The essential amino acid content in oat grain depends on the cultivar, however naked oat has higher amount of essential amino acids compared to hulled oat (Sterna et al., 2016). With regard to solubility, cereal proteins are grouped into four categories including albumins, globulins, prolamins and glutelins which are soluble in water, salt-water, dilute alcohol solutions and in acids or bases respectively (Ma & Harwalkar, 1984; Peterson, 1978 ; Robbins et al., 1971; Robert et al., 1985). Globulin proteins account for 70–80% followed by prolamins (4–15%), albumins (1–12%), and glutelins (< 10%) in oat grain (Nieto-Nieto et al., 2015). Most other cereals such as wheat, barley and rye contain higher proportion of alcohol-soluble prolamins, as their major storage protein (Klose & Arendt, 2012). Due to low prolamin and high globulin composition, oat protein is considered to provide a good balance of amino acids for humans and monogastric animals compared to many other cereals (M. A. Shotwell et al., 1990). Oat globulin proteins are similar to globulins in legumes, and are rich in glutamine and asparagine, although slightly low in sulfur-containing amino acids content (Colin et al., 2017). In terms of protein digestibility measured using simulated gastrointestinal digestion, oat protein has 86.58% digestibility which is slightly lower, yet comparable to that of soy protein which is 87.67% (Yuanqing et al., 2020).

Numerous studies have been carried out to review the functional properties of oat proteins. Ma & Harwalkar, (1984) evaluated the functional properties of oat protein fractions in terms of its solubility, emulsifying property, fat binding and water hydration capacities and foaming properties. The results suggest that oat protein exhibited better emulsifying and fat binding properties compared to that of soy protein. Furthermore, Nieto-Nieto et al., (2014) suggested that oat protein isolate (OPI) could be useful as a cost effective gelling ingredient upon enzymatic hydrolysis, which will address needs of the vegan and vegetarian food processing industry. Oat proteins which have been modified with enzymatic hydrolysis are more suitable to develop new food products with better protein functionality and they are emerging in the functional food market (Guan et al., 2007). Additionally, the possibility of using oat protein to supplement proteins in plant-based diets via incorporation in meat analogues has also been investigated (Malav et al., 2015). Even though it is well known that protein functionality is strongly dependent on the structural characteristics of oat protein, future studies are needed to fully explore how protein structural changes lead to functional differences. The structural characteristics of oat protein concentrate upon microbial fermentation has suggested its applicability as a substitute for yoghurt-type dairy food products (Brückner-Gühmann et al., 2019). Even though the exceptional high fat content in oat is appreciated for nutritional attributes it would impair the functional properties owned by oat. Therefore a recent study has suggested that selection of proper defatting method could substantially enhance the functional properties of OPI (Yue, Gu, et al., 2021a). However, with the growing interest in oat as a protein ingredient, there is a need to study the structure-function relationship, eco-friendly defatting methods, and versatile applications to increase the applicability of oat in food industry. This review is intended to provide a broad overview of uses of oat in the food industry, the role of oat as a novel protein ingredient in food applications, oat

protein structure and functionality and current and potential utilization in the food industry. Additionally, the knowledge gaps between oat protein structure, functionality and applications of oat proteins are also highlighted, while highlighting the need for future studies that explore how genotype and environment influence the techno-functionality of oat proteins.

2.3. Oat production, current state of usage and potential use as a protein ingredient

The genus *Avena* is comprised of about 70 species, and most of them are commercially grown (Gorash et al., 2017). Russia is known as the largest producer of oat (4.4 million tons) followed by Canada (4.2 million tons), and also it is the sixth highest consumed cereal worldwide (FAO STAT , 2019). In the northern hemisphere, oat is widely cultivated between the latitudes of 40°N and 60°N in North America, Europe and Asia (Forsberg & Reeves, 1995). In comparison to wheat and barley, oat has lower yield mainly due to the higher (25%–30%) husk weight percentage of an average oat grain (Rhymer, 2002). However, breeding efforts across North America have focused on developing oat cultivars that are higher in yield and disease resistance. Oat has the possibility to be grown in different soil types and after rye (*Secale cereale* L.), and is the most versatile cereal with regard to soil type on which it can be grown (Forsberg & Reeves, 1995). Oat is grown on any well-drained and fertile soil, under favorable temperature and moisture conditions. Additionally, oat can tolerate wet weather conditions and acidic soils better in comparison with wheat and barley. In fact, Givens et al., (2004) showed that growing oat is more cost efficient compared to wheat and barley because of its relatively higher resistance to foliar diseases, which leads to decreased requirements for agro-chemical and fertilizer. Numerous studies have indicated that there is a strong relationship between nitrogen supply from fertilizer and oat protein content. Fan et al., (2009) suggests the possibility of improving protein content and β-glucan contents by managing nitrogen supply to plants. Therefore, with the possibility of utilizing agricultural practices such as

nitrogen fertilization and with the availability of improved oat genotypes, oat has received renewed interest for its nutritional quality and functionality as a protein ingredient.

Oat has been used for human consumption over centuries as wet cooked porridge. Oat was introduced to North America in the 16th century when the first oat dehuller was invented (Menon et al., 2016). In later years, the technology to produce steel cut oat was developed, in addition to the technology for producing rolled oat. Currently, the most common end-product of oat is rolled (flaked) oat, used for typical breakfast porridge. Flaking or rolling produces desired changes in texture, which makes oat products more palatable and easier for domestic cooking, while also making the end-product more aesthetically pleasing (Webster, 1996). Moreover, quick-cooking oatmeal contains thinner oat flakes compared to that of regular oatmeal, which results in quick water absorption, thereby decreasing the cooking time (Decker et al., 2014). With increasing the awareness regarding benefits of whole grain consumption, the most common food preference of oat includes whole grain oat materials such as rolled oat, steel-cut oat, flour, ready-to-eat cereal as well as oat fractions such as bran.

As explained above, oat is generally processed as a whole grain product due to its softer groat, unlike wheat, which is not easily separated into endosperm, germ and bran fractions (Decker et al., 2014). Bread, cookies, muesli, granola bars and infant food are relatively new food products made from oat. Oat can also be useful as an extender in meat products such as sausages, and as a carbohydrate source for fermented beverages such as beer (Decker et al., 2014). Furthermore, oat is used as an ingredient of infant food due to its favorable nutritional attributes, hypoallergenic nature, palatable flavor, stability and low cost (Sterna et al., 2016). In most infant food products, oat is incorporated as a thickener, either in ready-to-eat form or in a dry form, which requires preparation prior to consumption (Ranhotra & Gelroth, 1995). Sandrin et al., (2017) suggested

that oat-based infant food can be easily prepared with oat flour using extrusion cooking. As such, oat-based low-cost infant formula has exerted better sensory and nutritional attributes compared to similar products that use milk and soy-based ingredients as main ingredients. Additionally, oat-based infant formula can be used for infants with lactose intolerance for satisfactory weight gain (Del Valle et al., 1981).

One of the advantages of using oat protein ingredients is its heat stability and OPC has a practical significance in food products which require heat treatments near 100°C (Brückner-Gühmann et al., 2019). Specifically, oat globulin is known to have higher heat stability in comparison to most plant proteins, making it an excellent ingredient for food that need structural stability after heat treatment (Ercili-Cura et al., 2015). Similarly, Ma & Harwalkar, (1984) reported that the exceptionally high degradation temperature for oat globulins is important in terms of food processing, as oat can be successfully utilized in products that need high thermal stability. A number of studies have been conducted to review the possibility of using oat for the development of nutraceuticals. Due to its antioxidant activity and high bile binding capacity, oat protein is a potential ingredient for the development of nutraceuticals (Shah et al., 2016). For example, curcumin loaded oat milk has been found to have high encapsulation efficiency (>90%) and bio-accessibility, suggesting the possibility of using oat milk as a delivery system for nutraceuticals (Zheng et al., 2021). Oat protein gels have also been used to prepare nutraceuticals loaded with probiotics, and have shown promising results in terms of protecting probiotics against deterioration in gastric conditions in an *in vitro* release study (Yang et al., 2017). Additionally, results indicated that OPI gels can resist pepsin digestion in the stomach and release bioactive compounds when examined in a simulated intestinal environment. Therefore, future applications of OPI gels include utilization as a carrier for sensitive compounds used in food applications.

Due to the increasing interest in plant-based protein ingredients, oat has gained renewed interest for its protein content and protein quality. In 2018, the global oat protein market reached to USD 48 million and is predicted to grow up to USD 63 million by the end of 2025, with a compound annual growth rate (CAGR) of 4.1% during 2019-2025 (360 Market Updates, 2018). With regard to usage and consumption, oat protein increased from 884,000 kg in 2012 to 1,398,000 kg in 2017 globally, with a CAGR of more than 9.6%. In terms of uses for oat protein in food applications, oat proteins have been known for better sensory properties in comparison to proteins of legumes and oil seeds (Boukid, 2021). Oat protein isolates and concentrates can be easily incorporated into pasta and bakery products to increase their protein content. Brückner-Gühmann et al., (2019) evaluated the possibility of using OPC for the development of a non-dairy yoghurt type product by fermenting OPC using a culture containing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. In this study, it was found that proteolytic enzymes can cleave proteins and release bioactive peptides, thereby enhancing the nutritional value of the product. Furthermore, acidification with lactic acid and gelling with the starch fraction contributed to improved sensorial quality in terms of viscosity and texture (Brückner-Gühmann et al., 2019). Given the increased demand for plant-based protein sources, meat analogues produced, from plant proteins have gained traction (Kyriakopoulou et al., 2018). The market for meat alternatives has long been dominated by soy, pea and wheat gluten proteins (Heusala et al., 2020). However, due to the presence of common allergens in soy and pea proteins, oat protein has a potential to play a significant role in the meat alternative market as a new protein ingredient. Angelis et al., (2020) studied the chemical properties and sensory attributes of meat alternatives produced using oat protein as a key ingredient and demonstrated that dry-fractionated protein can be used meat analogues of plant origin. One study states that OPC can be recovered during industrial production

of β-glucan, thereby oat has the possibility to be used as a cost effective protein source of plant origin (Brückner-Gühmann et al., 2019). Besides using oat protein in high protein pasta applications, yogurt, and plant-based meat applications, one of the most popular oat-based products that have dominated the market includes oat milk. Due to consumer awareness about the benefits of consuming oat milk and increasing popularity of vegetarian, vegan, and flexitarian diets, the global oat milk market reached USD 4.0 billion in 2020 and is predicted to grow with a CAGR of 9.8% (revenue based) by 2027 (Grand View Research, 2020). Bernat et al., (2015) suggested the use of oat milk is a promising alternative to dairy products, as it can be useful to combine probiotics to prepare fermented products resembling yoghurt. Furthermore, it is possible to adjust the viscosity, flow properties and the consistency of oat milk with temperature conditions during processing due to the presence of soluble dietary fiber and protein (Deswal et al., 2014). It has been found that oat milk prepared from 50% commercial oat flakes could significantly lower the total cholesterol in male subjects with moderate hypercholesterolemia, which indicates that oat milk could be a healthy alternative to traditional milk (Önning et al., 1999).

2.4. Processing of oat proteins: extraction techniques, benefits and disadvantages of methods and feasibility

Plant proteins in seeds or grains can be extracted in many ways. Ground and defatted cereal grains are successfully separated into protein fractions containing albumins, globulins, prolamins, and glutelins, extracted with water, salt solutions, alcoholic solution, and alkaline solutions respectively (Ma & Harwalkar, 1984; Peterson, 1978 ; Robbins et al., 1971; Robert et al., 1985). Previous studies have been conducted to extract OPC using wet-milling processes, using alkali solutions of different pH values (Cluskey et al., 1973). During wet-milling, protein is first solubilized in alkali solutions, and then isoelectrically precipitated under somewhat acidic

conditions, then dried at laboratory-scale through lyophilization or sprat drying. This approach is often referred to as AE–IEP (Ma, 1983). Moreover, when using the AE–IEP method, OPC with the highest protein content (72.1%) was obtained by using dilute alkali (0.015N NaOH) solutions at pH 9.0 according to (Ma, 1983). The OPC prepared through AE–IEP contained 65-70% total protein when solubilized at pH 9.5 as reported by Ma (1983). Yue, Gu, et al., (2021) differentiated between OPI and OPC as, proteins that are extracted using defatted oat flour and non-defatted oat flour, respectively. In this study, OPI contained more than 86.93% protein compared to that of OPC (<70.20%)when extracted through the AE–IEP method (Yue, Gu, et al., 2021a). In a more general context, protein isolates have higher protein content (>90%), compared to concentrates (59–75%) (Kriger et al., 2018; Ma, 1983).

During the preparation of OPI, oat flour is usually defatted prior to protein extraction as it contains high amount of fat (5-10%) (Yue, Gu, et al., 2021a). The type of defatting treatment used influences the lipid content of the resulting OPI as demonstrated in previous studies. For example, Wu et al., (1977) studied two different defatting methods to prepare OPI with 1-butanol and hexane, and results revealed a higher OPI yield (12%) resulting from butanol defatted flour compared to hexane-defatted flour (10%). According to Jiang et al., (2015), oat proteins produced using super critical carbon dioxide (SC-CO_2) as the defatting treatment, were found to have 62% protein, 17% starch, 3% fat and 2% dietary fiber. Studies were conducted to evaluate functional properties including solubility, foaming and emulsification properties of oat flour defatted using SC-CO_2, where the results indicated SC-CO_2 extracted flour exhibited better functional properties in comparison to non-defatted oat flour (Konak et al., (2014). Although the AE–IEP approach is commonly used for oat protein extraction, it is labor intensive, time consuming and environmentally problematic (due to use of more water, organic solvents and energy consumption)

and can potentially denature proteins due to repeated centrifugation and pH adjustment (Yue, Zhu, et al., 2021a). In industrial settings, the AE–IEP process is not very feasible as it involves consumption of huge amounts of water and energy, particularly for the mixing and drying steps; however the OPI produced through this method is of high purity (>80%) (Schutyser & van der Goot, 2011).

Schutyser & van der Goot, (2011) studied the potential of dry fractionation methods, as an energy efficient method for plant protein extraction in comparison to wet methods and indicated that, OPC with 20-27% protein content could be extracted by dry fractionation through milling followed by air classification methods. In another study SC-CO_2 defatted oat flour was separated into fractions containing β-glucan, protein and starch concentrates by milling followed by sieving and air classification, while the fraction obtained through supercritical extraction, comprised of the lipid concentrate (Kaukovirta-Norja et al., 2010). As explained above, there is a need to develop methods that are energy and cost-efficient and industry-feasible for the extraction of OPI and to address this need, a patented method was developed by Kaukovirta-Norja et al., (2010). The method does not use solvent extraction or heat treatments, which results in OPI with good functionality.

Nałęcz et al., (2017) described a modified method for oat protein extraction, which is based on solubility differences among the protein fractions. In this method, an extraction buffer composed of 6 M urea, 1% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 0.5% (w/v) dithiothreitol and 1 mM phenylmethylsulfonyl fluoride was used for protein extraction from oat flour. The authors suggest that this method can be useful for the separation and quantification of oat protein, although protein yield is lower due to multiple purification stages (Table 2.1.) involving solid phase extraction using water, acetonitrile and trifluoroacetic acid. Deep Eutectic

Solvents are usually made of two components, including a hydrogen bond donor and an acceptor (Serhan et al., 2016). In this method, water acts as a major component of the two phases to provide a mild environment which protects the structure of biomolecules, such as proteins (R. tao Zhao et al., 2020). Xu et al., (2014) conducted a study to extract Bovine Serum Albumin (BSA) using four kinds of ChCl/alcohols-based DES and reported that the use of DES leads to high extraction efficiency of BSA (98.71%). Bai et al., (2017) used DES for the extraction of collagen proteins from cod skins, to be used in food, biomedical, cosmetic, and pharmaceutical applications, which also showed high extraction efficiency (>91.57%) and high purity (>93.14%). Novel methods such as extraction through DES are promising for its utilization in the plant protein realm, however, the structural and functional characteristics of protein extracted using DES are yet to be analyzed for their functionality in different food systems.

Table 2.1.Oat protein extraction techniques: Advantages and disadvantages

Technique	Advantages	Disadvantages	References
wet-milling processes - alkaline extraction followed by isoelectric precipitation	Provides a protein isolate with high protein content (>90%)	Usage of huge amounts of water and energy	(Kriger et al., 2018 ; Yung Ma, 1983)
Dry fractionation methods	Energy efficient method	Yield less protein content (<30%)	(Schutyser & van der Goot, 2011)
Protein isolation with Deep Eutectic Solvents (DES)	Sustainable method that utilizes biodegradable components and solvent	Low protein yields due to multiple purification stages	(Nałęcz et al., 2017 ;Zainal-Abidin et al., 2017)

2.5. Oat protein composition and structure

Oat contains 12-20% protein by weight comprising of 50-80% globulin, 4-15% prolamin, 1-12% albumin and less than 10% glutelin (Klose & Arendt, 2012). Oat albumin consists of the metabolically active water-soluble protein fraction, whereas the oat globulin fraction exhibits low water solubility near neutral and acidic pH conditions (Jiang et al., 2015). According to Klose & Arendt, (2012), the globulin fraction deriving from the endosperm protein bodies are soluble only in 1 mol/L salt solutions, while globulins located in the cell wall are more soluble in water. The

prolamin fraction of oat protein is insoluble in water, while perfectly soluble in alcohol (Peterson, 1978). The glutelin fraction of oat protein shows poor water solubility (<20%) near pH range between 3 and 8, whereas the maximum water solubility (about 90%) can be observed at approximately pH 10.5 (Ma & Harwalkar, 1984).

Recent studies on amino acid profile of naked oat demonstrated that oat has more hydrophilic amino acids than hydrophobic residues (Yue et al., 2021). With regard to amino acid composition, oat contain higher amounts of glutamic acid and leucine accounting for 24.7% and 8.1% of the total amino acids in OPI, respectively. However, with respect to essential amino acids (EAA) in OPI, 32.3% of total amino acids are consist of EAA including leucine, isoleucine lysine, methionine, threonine, valine and phenylalanine. (G. Liu et al., 2009). Even though the EAA content of OPI is significantly lower than that of soy protein (60.3%) (Table 2.2.), OPI has commercial importance in developing breakfast cereals without most common allergens found in legumes including soy proteins (Gorinstein et al., 2002). Advances in biotechnology have the potential to improve the nutrient value of cereal grains by increasing the levels of EAA, specifically lysine (Kawakatsu & Takaiwa, 2018). With reference to protein secondary structure, the morphology of OPI was first studied using Fourier transform infrared spectroscopy (FTIR) with OPI extracted from a high-protein oat cultivar (G. Liu et al., 2009). The results indicated that the secondary structure of oat protein isolate powder consists of approximately 19% α-helix, 7% β-turn, and 74% β-sheet. The study also shows that, below 0.5 mg/mL of protein concentrations, self-assembled individual protein structures appear as disk-like or ellipsoidal shapes.

The most metabolically active proteins are comprised of enzymes, and in oat they can be categorized as maltase, α-amylase, proteases, lichenase, phenoxyacetylase, hydroxylase, phosphatase, tyrosinase and lipase (Lásztity, 1998). The presence of lipase can be attributed to oat

containing exceptionally higher fat content compared to most other cereals. Oat also contains proteinase inhibitors, which are found in most seeds and are considered to be anti-nutritional factors (Klose & Arendt, 2012). However, Mikola & Mikkonen, (1999) showed that both trypsin and chymotrypsin inhibitors present in oat could be destroyed by either low pH (pH of human stomach) or high temperatures above 60 °C, which is often used during oat processing. Oat albumins have the highest levels of lysine, alanine, aspartic acid, glycine and threonine and the lowest levels of glutamine-glutamic acid in comparison to other oat protein fractions (Lásztity, 1998). Oat contains considerably higher amounts of tryptophan compared to other cereals, and is found mostly in albumin and glutelin fractions (Klose & Arendt, 2012). The major oat albumin protein fractions show molecular weights of 15000, 22000, and 36000 and 6000 for minor constituents, with isoelectric points between 4 and 7.5 (Ma & Harwalkar, 1984).

Oat globulin comprises of the salt soluble fraction of proteins as stated earlier (Lásztity, 1998). In oat, the globulins are located inside the protein bodies that are 0.3 to 5 μm in diameter (Mäkinen et al., 2016). Oat globulin is high in glutamic acid/glutamine, aspartic acid/asparagine, tyrosine, and phenylalanine, but somewhat low in the basic amino acids, lysine and histidine (Peterson, 1978). The globulin fraction of oat is a mixture of different polypeptides of 3, 7, and 12S fractions, when extracted with 1 mol/L NaCl (Burgess et al., 1983). In oat, 12S globulin is the major globulin fraction, which has a quaternary structure similar to that of soy (Klose & Arendt, 2012). With regard to structure, the 12S globulin protein fraction is a 320,000 Da hexamer, in which the subunits are bonded by non-covalent forces (Li & Xiong, 2021). Peterson (1978) also studied the oat globulin fraction with regard to its molecular weight, subunit composition and amino acid balance and reported that oat globulin consist of two major subunits of 21,700 and 31,700 Da, referred to as α and β subunits respectively. This is in agreement with the findings of Mäkinen et

al., (2016) who reported that oat 12S globulin is assembled as α and β subunits. Furthermore, according to Klose & Arendt, (2012), α- and β-subunits are held together by disulfide bonds forming a dimer with a molecular weight of about 54,000 Da; thereby, the 12 S globulin is a hexamer which is made of six dimers. The 7S globulins are composed of 55,000 Da polypeptides, while 3S fraction consists of two constituents, having molecular weights of 15,000 and 21,000 Da. The isoelectric point for globulin proteins are at pH 3.5 (Peterson, 1978). It has been found that temperatures between 140°C to 240°C has the possibility to alter the secondary structure of oat globulins, as demonstrated by decreasing of β- sheet and β- turn content, and increasing α- helix coil contents (He et al., 2021). Oat is the only cereal that has globulin fraction as the major storage protein, similar to legumes such as soy (Lásztity, 1998). Furthermore, soy globulins known as glycinin proteins, belong to the 11S globulin family (Adachi et al., 2003). The similarities between oat globulin and soy glycinin with regard to structure and molecular weight indicate the use of oat as a substitute for soy, which is one of the Big 8 allergens (Ercili-Cura et al., 2015).

Oat has reproducible polymorphism in the prolamin fraction and, prolamin and glutelins are collectively known as avenin proteins (Klose & Arendt, 2012). Prolamin is the alcohol soluble fraction of oat which has shown maximum extractability in 45% (w/w) ethanol (Kim et al., 1978). The glutamic acid and glutamine are higher in the prolamin fraction of oat protein, whereas low amounts of proline is found compared to the prolamins of other cereals (Peter R. Shewry, 1999). The prolamin fraction contains the lowest amount of lysine, among oat protein fractions (Draper, 1973). The low proline and high leucine and valine content in oat prolamin makes it similar to prolamins of rice, millet, and maize (Klose & Arendt, 2012). The conformation of prolamin is largely globular and this protein fraction is high in α-helix with more proline containing repeats forming β-reverse turns (Shewry, 1999). With regard to the cellular structure of the oat grain,

prolamins are deposited in protein bodies located in the endosperm and are not found in the aleurone layer (Pernollet et al., 1982). Prolamin is known to be synthesized in the rough endoplasmic reticulum and then transported to be deposited in protein bodies (Burgess & Miflin, 1985). The molecular weight of prolamin proteins constituents has been reported as 23,500 and 15,500 with isoelectric points between pH 6-7.5 and 9, respectively (Ma & Harwalkar, 1984).

The oat glutelin fraction is extracted with either acids or bases, and Robert et al., (1985) reported that two protein fractions can be extracted following the extraction of albumin, globulins and prolamins. These findings are in agreement with those of Ma & Harwalkar, (1984) who indicated that oat glutelins are composed of a major subunit and a minor subunit with molecular weights of 18,000 and 10,000 respectively. The glutelin fraction contains the highest content of aspartic acid, tyrosine and tryptophan compared to the other protein fractions of oat (Lásztity, 1998). The glutelin fraction has isoelectric points in both acidic (pH 4.5-6.5) and alkaline (pH 9-10) regions (Ma & Harwalkar, 1984). Studies have also shown that the glutelin fraction has typically been difficult to completely solubilize and mostly gets mixed with other fractions, such as albumin, globulin and prolamin when separating via electrophoresis. Klose et al., (2009) also report that the glutelin fraction co-migrates with prolamin and globulin fractions. The results also indicated that protein content of the glutelin fraction decreased during malting process because of the cleavage of the proteins into small peptides and amino acids (Klose et al., 2009). Oat glutelin peptides resulting from hydrolysis with alcalase proteases have been assessed for their antioxidant activities, and results revealed that a peptide with 7 amino acid residues has the highest antioxidant activity (Ma et al., 2017).

Table 2.2. Essential amino acid composition of whole grain oat, rice, wheat, soy and pea (g/100 g protein)

Essential amino acid	oat[ab]	rice[c]	wheat[c]	soy[de]	pea[f]
Isoleucine	3.6	3.8	3.7	5.2	3.3
Leucine	7.8	8.2	6.8	7.9	6.6
Lysine	4.3	3.7	2.8	6.2	6.8
Threonine	3.2	3.4	2.9	3.2	3.6
Valine	5.3	5.8	4.4	5.3	3.9
Phenylalanine	5.7	4.8	4.7	9.0	4.2
Histidin	2.3	2.4	2.3	3.0	2.5
tryptophan	0.9	1.3	1.1	1.6	0.9
methionine	1.3	2.1	1.2	0.8	1.0

Sources : [d]Gorinstein et al., 2002; [f]Leterme et al., 1990 ; [a]Liu et al., 2009 ; [b]Pettersson et al., 1996 ; [c]Shewry, 2007 ; [e]Torún et al., 1981

2.6. Techno-functional properties of oat proteins and their application in food industry

Oat protein functionality have been studied in terms of solubility, foaming ability, emulsifying properties, water holding capacity, fat binding capacity and gelling properties (Ma, 1983) (Figure 2.1.). Solubility of a protein is a key factor that determines all other functional properties it can possess (Kinsella, 1976). Oat albumins have high solubility over a wide pH range, while globulins show the highest solubility only at acidic and alkaline pH. (Ma & Harwalkar, 1984). However, all oat protein fractions are soluble at alkaline pH, suggests the ability of alkaline extraction of oat proteins (Ma & Harwalkar, 1984). However, oat globulin, which is the major protein in oat, exhibits low solubility at neutral pH, presenting drawbacks for its use in aqueous food systems (Jiang et al., 2015).The chemical, physical and enzymatic methods have been used to enhance the functional properties of oat proteins. It is also possible to enhance oat protein solubility by

modification methods such as deamidation and succinylation, where succinylation improved the solubility of unmodified OPI from 22.9% to 86.8% (Mirmoghtadaie et al., 2009). Furthermore, it has been found that deamidation with food grade protein-glutaminase can double the water solubility of oat protein (Jiang et al., 2015). Proteins with improved water solubility facilitates the development of high protein beverage applications and high solubility aids in producing a homogenous products with good shelf life (Konak et al., 2014). The higher denaturation temperature (105.64°C) of OPI and β-glucan conjugates revealed that the thermal stability of OPI is improved by stretching the secondary structure (Zhong et al., 2019). This superior heat stability of oat proteins could be useful in formulation of aqueous solutions of oat protein such as energy drinks, which needs to be processed at higher (>100°C) temperatures.

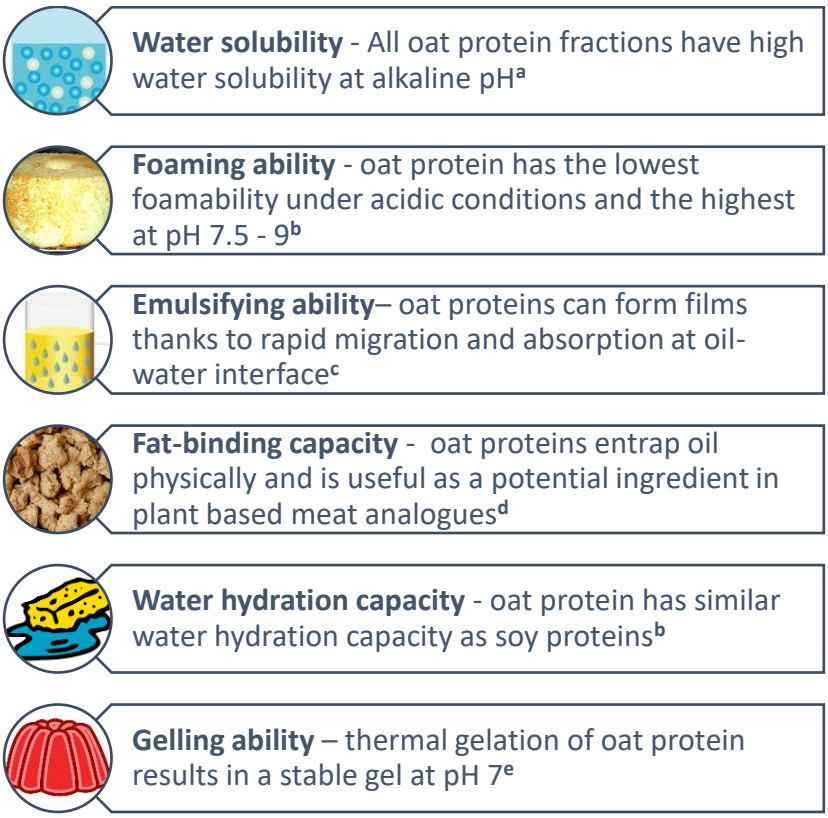

Figure 2.1. Summary of techno-functional properties of oat proteins

Sources : [d]Kinsella, 1976; [c]Konak et al., 2014; [a]Ma & Harwalkar, 1984; [b]Ma, 1983; [e]Nieto-Nieto et al., 2014

Protein foams are found in many food including nougat, meringue and angel food cake, where a protein incorporates the air phase to increase the volume, and maintain its stability following the processes such as mixing and heating (Foegeding et al., 2006). Konak et al., (2014) evaluated the functional properties of oat protein defatted through SC-CO_2 which revealed that basic pH conditions enhance the emulsifying and foaming properties. The relative stability of foams increased from 69% to 80%, when the pH was increased from 4.5 to 10.5. The increased stability of foam is attributed to increased solubility of oat protein at higher pH. Egg white has long been used in many food applications due to its capacity to form a highly stable foam with high volume.

However, as demonstrated by Konak et al., (2014) SC-CO_2-extracted oat is suggested to be used in beverages where foamability and foam stability play important roles. In a different study, OPI showed similar foaming capacity compared to lupin protein isolate and, two times greater foam stability (Mohamed et al., 2009). Kaukonen et al., (2011) studied the foaming properties of water extracted, hexane extracted, and SC-CO_2 extracted oat, and showed that SC-CO_2 extracted oat protein has excellent foaming capacity mainly due to the selective removal of nonpolar lipids and presence of tryptophanin proteins, which is considered as the major foam-active protein in oat. However, it is interesting to note that reduced foaming ability in hexane defatted flour may be due to the absence of tryptophanin protein, which is co-extracted along with the lipid fraction because tryptophanin is a lipid-binding protein. On the other hand, when using kilned oat to prepare OPI through water extraction, low foamability is attributed to the presence of non-polar lipids (Kaukonen et al., 2011). Moreover, SC-CO_2 extracted oat contained a high proportion of soluble proteins, compared to kilned oat, suggesting that heat treatments such as kilning can reduce solubility of proteins due to the formation of clumps of denatured proteins and thereby reduce protein functionality (Ma & Harwalkar, 1984). Furthermore, Brückner-Gühmann et al., (2018) evaluated the foamability of OPI hydrolyzed with trypsin, and showed that stable and fast forming foams can be prepared from hydrolyzed OPI at pH 4 and 7. Brückner-Gühmann et al., (2018) also shows that the air-water dispersion can be stabilized with hydrophobic interactions among the protein molecules leading to improved foam strength. The better foaming properties of OPI at neutral pH can enhance the applicability of OPI to replace animal protein such as egg white, however more studies are required to better understand the oat foam stabilization mechanisms of oat proteins in food systems (Brückner-Gühmann et al., 2018).

The surface active properties of a protein are influenced by different intrinsic factors such as protein structure and extrinsic factors including pH, ion concentration, temperature and protein content (Konak et al., 2014). The OPI has the lowest emulsifying ability at pH 5 which was measured by sample dispersions at different protein concentrations (Guan et al., 2007). This agrees with the results of Yungs Ma, (1983), who found that emulsification of the oat protein is strongly dependent of the solubility. Guan et al., (2007) also suggested that the solubility of a protein is the determinant for film forming ability due to rapid migration and absorption at oil-water interface. The albumin fraction of oat protein has higher emulsifying activity index at pH near 3.0-4.5 and also at 9.5 than at neutral pH conditions, which explains how protein solubility influences emulsifying ability. Liu et al., (2009) explained the association between oat protein secondary structure and self-assembly properties at the air/water interface and demonstrated that increases in protein concentration can enhance the self-assembly by aggregation of neighboring molecules. In a different study, OPI showed about 30% higher emulsifying activity than that of lupin protein (Mohamed et al., 2009). Moreover, studies found that enzymatic modification of OPI results in increased solubility near pH 5, at which most protein based beverages are formulated (Guan et al., 2007). Emulsion stability is another parameter to be considered for the use of OPI in food applications. It has been found that emulsion stability of SC-CO_2 extracted OPI could be influenced by heat, addition of salts and sucrose (Konak et al., 2014). Additionally, deamidation of oat protein was found to enhance the stability of emulsions, mainly due to uniform droplet size distribution (Jiang et al., 2015). It was also found that deamidation by the food-grade enzyme glutaminase, increases the flexibility of oat protein secondary structure, thereby influencing its ability to form a cohesive film at the oil-water interface due to increased surface activity (Jiang et al., 2015). Zhong et al., (2019) evaluated the effect of heat treatment under controlled conditions

as a method to enhance the functional properties of OPI, by conjugating it with β-glucan from *Pleurotus ostreatus* (as edible mushroom) via the Maillard reaction. The solubility and the emulsifying ability of OPI were improved after the Maillard reaction, attributing to changes in protein structure, which indicated changes in secondary structure caused by decreasing α-helix and β-sheet content and increasing β-turn and random coil content. In the context of Maillard-induced reactions for protein modification, cysteine and lysine are the dominant Maillard reaction sites in OPI, and the comparatively high content of these amino acids in OPI increases the likelihood of successful Maillard reaction-associated protein modifications (Klose & Arendt, 2012). From an application perspective, improved emulsification ability and emulsion stability have a significant commercial importance in developing low-fat products including, salad dressing and different snacks using oat protein (Mohamed et al., 2009).

Fat-binding capacity is a major determinant of plant proteins for utilization as meat replacers and extenders (Ma & Harwalkar, 1984). Kinsella, (1976) reported that the mechanism of fat absorption by protein is attributed mostly to the physical entrapment of oil. However, a number of factors could affect the fat-binding capacity, such as protein content, surface area, hydrophobicity of proteins, charge, and topography and liquidity of the oil (Ei-Adawy, 2000). Although soy has commonly been used as a meat extender and meat alternative in some applications, the use of soy has become problematic due to presence of food allergens (Asgar et al., 2010). Oat protein, on the other hand, is not recognized among the Big 8 allergens, making it an attractive alternative to soy for use in the plant-based meat industry. The oat albumin fraction has the highest fat-binding capacity (2.8 mL/g) as demonstrated by Ma & Harwalkar, (1984), whereas the fat-binding capacity of globulin, prolamin and glutelin were reported as 1.6 mL/g , 1.7 mL/g and 2.1 mL/g, respectively. However, it is important to note that the major protein fraction globulin, has good fat

binding capacity, indicating that OPI can be used in food applications such as meat analogues. Siu et al., (2002) evaluated the fat-binding capacity of globulin after polymerization using microbial transglutaminase and the results showed improvements in fat-binding capacity, water hydration capacity and foaming ability compared to native oat globulin protein. It has also been suggested that modification methods such as succinylation could improve the fat-binding capacity of OPI, together with water holding capacity (Mirmoghtadaie et al., 2009).

The water hydration capacity (WHC) of a protein is defined as the potential to hold its own and added water while it is subjected to different processing conditions such as, pressing, centrifugation, or heating (Joseph, 1997). Water hydrating capacity a major functional property of proteins as the moisture absorption has a great impact on the overall quality and sensory attributes of the final product (Walters et al., 2018). The WHC of oat protein concentrate (2.7 mL/g) is similar to that of soy protein isolate (2.5 mL/g), yet higher than that of gluten (0.98 mL/g) (Ma, 1983). In a different study, Mirmoghtadaie et al., (2009) showed the WHC of isoelectrically precipitated OPI without any physical modifications absorbed 1.27 g moisture per gram. Considering the different oat protein fractions, albumins (2.4 mL/g) and glutelins (1.9 mL/g) were found to have water hydration capacity higher than that of globulins (0.8 mL/g) and prolamins (0.9 mL/g) (Ma & Harwalkar, 1984). Furthermore, it is suggested that high WHC of oat glutelins could be due to the low solubility of this fraction. As for the albumin fraction, carbohydrate components in the fraction and high solubility may contribute to higher WHC. Siu et al., (2002) showed that use of transglutaminase to modify the globulin fraction, could exert better WHC than native globulin proteins. Furthermore, the results indicated that increases in WHC were due to the formation of large clusters of protein molecules, thereby increasing the ability to swell and take up water. Similarly, WHC of oat protein was improved by linoleate and trypsin treatments, (Ma &

Wood, 1987). Moreover, OPI is important in the alternative meat industry since the relative high WHC of OPI makes it a good candidate to be used as a gelling agent in food applications. (Nieto-Nieto et al., 2014).

Gelation can be regarded as a major functional property, which is key to the structure of food products (Asgar et al., 2010). In commercial food applications, egg white and gelatin are the most commonly used gelling ingredients. Thermal gelation of globular proteins involve unfolding of peptide chains by heat, which leads to exposure of hydrophobic amino acid residues, followed by re-arrangement and irreversible aggregation via disulfide bridges, hydrogen bonds, hydrophobic and/or van der Waals interactions (Nieto-Nieto et al., 2014). Native oat proteins form a polymer gel at neutral pH, whereas the flavourzyme, alcalase, pepsin and trypsin treated hydrolysates form gels at pH 9 (Nieto-Nieto et al., 2014). Therefore, partial hydrolysis has been used to enhance the WHC and thereby, the gelling ability of OPI (Nieto-Nieto et al., 2014). As for the gel formation mechanism, it has been suggested that a small portion of oligomeric oat globulin form soluble aggregates at 110°C, and with further heating these aggregates would either disassociate into subunits or further aggregate and precipitate (Ma & Harwalkar, 1987). Insoluble aggregates can be formed by protein-protein interactions of partially denatured subunits or by the disruption of the quaternary structure of oligomers (Ma & Harwalkar, 1987). The oat 12S globulin structure is similar that of soy glycinin that exhibits superior gelling ability, therefore, oat protein also has the ability to be a promising gelling agent (Nieto-Nieto et al., 2014). However, Ma & Harwalkar, (1987) suggested that there is a significant difference in heat coagulation of oat globulin and soy glycinin even though they show structural similarities. Due to excellent WHC and gelling properties, OPI can be used as a low-cost gelling agent to replace the use of animal protein to support structure in food and as an alternative to allergens such as soy.

2.7. Genotypic and environmental effect on oat protein quality: why is it important to study G × E interactions?

The G × E interaction for characteristics such as grain yield, groat percentage, β-glucan, protein content of oat and their genetic correlations were studied using two genetic populations in multi-location trials from 2010 to 2012 in eastern Canada (Yan et al., 2016). The results of the study indicated a negative correlation between groat percentage and β-glucan content, a negative correlation between grain yield and protein content, a positive correlation between β-glucan content and oil content, and a negative correlation between grain yield and groat percentage, which was not as prominent as the previously identified correlations. Although there is a negative correlation between grain yield and protein content, oat cultivars with high yield and relatively low protein content can be used in the oat milling industry (Yan et al., 2016). In another study, Jenkins (1969) reported that genotype is the major influencing factor with regard to fat content of oat, in comparison to growing environment. Mut et al., (2018) evaluated the adoptability of twenty-five local oat cultivars to six new environments across Turkey, and studied the characteristics such as, thousand-grain weight, test weight, groat percentage and proximate composition. The results of this study indicated that variance resulting from environmental components are much larger than that of genotypic variance for test weight, grain yield, thousand grain weight, as well as protein, starch, and ash content. In contrast Doehlert et al., (2001) investigated the influence of genotype and environment on grain yield and quality of 12 oat genotypes cultivated within 3 years, at four locations in North Dakota. The results showed that test weight, groat percentage, groat weight, protein content, and β- glucan content had similar influences from environment and genotype, whereas groat lipid was strongly dependent on genotype alone, which is not in agreement with the findings of Yan et al., (2016) as well. Traits that are influenced by genetics can

be manipulated by plant breeding, although this is a complex task that is time consuming, whereas traits that are impacted by environmental factors such as weather and soil conditions cannot be modified through such methods (C. Rhymer et al., 2005).

Information about what environmental factors affect the G × E interaction, help plant breeders to understand the nature of these interactions for developing stable cultivars that can tolerate climatic and edaphic variation (Gullord & Aastveit, 1987). Historical oat cultivars originating from Canada, Europe and New Zealand are usually not grown due to their low yield, however such cultivars show potential to be improved for better yield (Stevens et al., 2004). Moreover, cultivars that were introduced into Asia over the past 20 years, have a particular importance in feeding livestock across the regions bordering the Himalayas, where they are used as green feed. However, due to considerable G × E effects noted across latitudes, research has suggested that such cultivars need to be tested in different growing regions as they may have the potential to be used as a source of human nutrition (Stevens et al., 2004). Considering environmental impacts on oat physiological development, Doehlert et al., (2001) found that warm spring weather conditions as well as cool summer weather conditions without severe rains in grain filling period could lead to better yield in terms of grain quality and quantity. The grain yield showed variation between the environments mainly through the effect of rainfall and the temperature fluctuations (Mut et al., 2018). Hellewell et al., (1996) studied the influence of day temperature, night temperature, the day-night temperature differential during grain filling period on oat grain yield. The results revealed that quality characteristics are largely influenced by the day temperature rather than night temperature and the day-night temperature differential. Additionally, it has been found that oat quality and yield are mostly influenced by heat, soil moisture and also by light radiation in different growth stages of oat crops (Hellewell et al., 1996). With regard to nitrogen content during oat cultivation,

studies have shown that nitrogen fertilizer application has a positive effect on protein content in oat kernels (Fan et al., 2009). The results from the analysis of straw samples suggested that high grain yielding cultivars take up more nitrogen per unit area from soil and translocate relatively higher nitrogen content to the grain from vegetative tissues, compared to the cultivars with low grain yields. Jenkins (1969) analyzed the relationship between nitrogen content and yield in four oat cultivars and indicated that yield of crude protein is closely related to grain yield therefore, higher yielding cultivars may take up more nitrogen per unit area. Nitrogen fertilization can significantly maximize grain yield and increase protein concentration in oat kernels (Gorash et al., 2017). This demonstrates that the protein content has the ability to be manipulated (to a certain extent) by the selection of oat cultivar, growing environment and by suitable fertilizer application. With regard to variations caused by genotype effects, Buerstmayr et al., (2007) studied the agronomic and grain quality characters of 120 oat genotypes of worldwide origin, in three replicated field experiments in Austria and Germany. According to results, substantial genetic variation and high heritability were observed for all characteristics such as grain yield, thousand kernel weight and test weight, whereas thousand-grain weight was strongly influenced by genotype, with lower variations among the treatments or environments (Buerstmayr et al., 2007). In another study, thirty-three oat genotypes were grown in Idaho throughout three consecutive years (1999–2001), where quality characteristics such as, yield, kernel physical characteristics, heading date, and concentration of groat protein, β-glucan, oil, tocols, and avenanthramides were evaluated (Peterson et al., 2005). The study showed that genotype is the main factor impacting β-glucan and protein concentrations in hulled oat cultivars. Moreover, Mut et al., (2018) also reported that the growing geographical location influences the protein contents of oat cultivars.

Many farmers prefer conventional/covered/hulled oat over naked oat mainly due to the limited market for naked oat and the price similarity between conventional and naked oat (Gorash et al., 2017). It has been found that there are significant G × E interactions affecting husk content, test weight and protein content of oat as evidenced previously. Due to the availability of good quality proteins, oat is considered as an important crop (Doehlert, 2002). The protein content of oat grain is a critical parameter to consider before choosing starting materials to manufacture OPC or OPI (Yue, Gu, et al., 2021a). Furthermore, the protein content of oat is particularly significant in terms of grain quality characteristics that are considered when using grains for the production of OPI. When considering the whole oat kernel, the thick hull reduces the nutritional and economical value of oat grain as animal feed and adds extra cost for transporting. However, breeding programs have focused on developing highly productive naked oat cultivars and also reducing hull percentage in conventional oat which presents benefits to oat millers (Gorash et al., 2017).

Oat breeding practices are mainly focused on identifying quality oat genotypes with high quality to provide parental genotypes of excellent quality, and at the same time develop superior genotypes compared to the existing ones (Mut et al., 2018). According to Yan et al., (2016), the biggest challenge in oat breeding is not the G × E for single quality traits, or the adverse association between grain yield with any quality trait, but combining grain yield with the best package of quality. High protein oat genotypes that are more stable across different environments can be a better choice as a parent for the high-protein oat, used for OPI production worldwide (Peterson et al., 2005). Further studies are needed to develop high-yielding cultivars that present consistent quality characteristics, which are adaptable to a wide range of environmental conditions. As explained in the previous sections, the techno-functional properties of protein are the major determinants of the quality attributes of the final product. Additionally, the functional properties

of protein fractions are influenced by the protein structure, composition and configuration, which are impacted by G × E interactions (Kinsella, 1976). There have been many studies that assessed this interaction from a breeding perspective, especially in wheat. Kaya & Akcura, (2014) studied the influence of genotype, environment, and G × E interactions regarding variations in wheat grain yield and other quality traits. The study showed that environment is the main factor controlling test weight, grain yield and thousand kernel weights, indicating 63%, 70.2% and 78.5% of the total variance, respectively. From an end-use quality perspective, wheat breeders have studied methods to develop wheat cultivars with better breadmaking properties, taking into account the impact of environmental and genetic factors. Considering environmental factors that affect the breadmaking quality of wheat, Guzmán et al., (2016) evaluated the effects of drought and heat stresses on breadmaking quality of durum wheat and bread wheat and, the results indicated that heat stress causes an increase of extensibility in both wheat, favoring the breadmaking quality of durum wheat, showing the potential of durum wheat in bakery applications. Several studies have been conducted to evaluate the impact of G × E on wheat protein composition and thereby bread making quality characteristics. Since wheat end-use quality is impacted by gluten proteins, Malalgoda et al., (2018) studied the association between quality traits and protein fractions of wheat cultivars that were released from1910 to 2013, and indicated that farinograph peak time and stability improved over the years, however, a significant difference was not found in the total protein content during the last 100 years of wheat breeding.

Even though many breeding approaches have focused on improving the grain quality and disease resistance, there are fewer breeding efforts undertaken to improve oat protein functionality. Many studies have focused on developing oat cultivars with improved winter-hardiness, pest and disease tolerance, milling quality and health benefits. However, there have been some studies focused on

enhancing oat protein content through breeding practices (Jackson, 2012). As previously mentioned, naked oat is less popular among farmers than hulled oat. Therefore, breeding practices have been applied to produce high quality naked oat grains with 10.59 % (dry basis) protein content that is suitable for many food purposes (Batalova et al., 2016). More genomic assisted breeding tools are needed to fully explore how breeding practices affect the specific functional properties of oat proteins and thereby to enhance the functionality of oat proteins. In comparison to oat, pulses have been more extensively explored for protein structure and functionality. For example, K. Liu, (1997) described that the functional properties of soy protein could be altered by changing the 11S to 7S protein ratio. Such changes in protein composition could result in significant changes in thermal stability, gelling properties and emulsification characteristics (Kitamura, 1993). As such, it is possible to manipulate the functional properties of oat proteins through breeding for specific protein characteristics, while considering the impact of G × E interactions. However, future studies are needed to explore G × E interactions on quality characteristics of oat proteins, to extract superior OPI with excellent functional properties from oat grains and to produce protein ingredients of consistent quality.

2.8. Conclusions

Oat contains approximately 12- 20% protein content, which varies the genotype and growing environment. The major protein fractions in oat are comprised of globulin, albumin, prolamin and glutelin, where globulin accounts for 70–80% of the total protein content. With regard to nutritional attributes, OPI contains essential amino acids, which represent about 32.3% of total amino acid content in OPI. In terms of protein quality, OPI is comparable to that of soy protein isolate, where OPI has 86.58% digestibility which is slightly lower than soy protein (87.67%). Oat protein is primarily extracted either as OPI or OPC where, OPI has higher protein content (>90%),

compared to OPC (59–75%). Furthermore, OPI is extracted in alkali solutions (at pH 9 approximately), followed by isoelectric precipitation at pH 5- 5.7. However, there is a need to explore novel and eco-friendly processing methods for the production of OPI and OPC, which require less resources. The techno-functional properties of OPI include foamability, emulsifying properties, water, and fat binding capacity, and gelling properties, indicating that oat proteins have the potential to replace soy and animal protein in food applications. Additionally, the major fraction of oat protein, globulin exhibits higher heat stability in comparison to most plant proteins, making it a potential ingredient for food that need structural stability after heat treatment. As oat protein content and composition are influenced by genotype and environmental factors, a good understanding of G × E impacts on oat protein is needed to successfully utilize OPI in food applications, without variations in end-product quality. Overall, the review highlights critical factors that are crucial for increased utilized of oat protein in different food systems and demonstrates the importance of understanding the impact of different factors including extraction conditions and G × E, in order to successfully develop OPI-based products of excellent nutritional quality, flavor and aesthetics.

2.9. Most recent updates in oat protein research

The goal of the section is to summarize the most recent findings related to oat protein research. In a recent study OPI was produced from oat flakes through wet extraction after enzymatic pretreatment to eliminate starch and non-starch polysaccharides using α-amylase and xylanases, which resulted in protein concentrations of up to 86% in dry matter (Sargautis & Kince, 2023). Furthermore, the authors reported that extraction conditions pertaining to the enzymes used had no effect on the amino acid composition, although a reduction in aqueous solubility of recovered proteins was observed. Protein modifications with chemical, physical and enzymatic approaches

to enhance the functionality of oat protein is an emerging and important area of research. Another study was carried out to investigate the effect of roasting and amylase hydrolysis pretreatments on the structural and functional properties of OPI extracted through wet extraction and that the results showed, even though pretreatments could enhance the surface hydrophobicity and thermal stability of OPI, pretreatments can diminish aqueous solubility and emulsifying properties of OPI (Wang et al., 2022). According to Cheng et al. (2022), dynamic high-pressure micro fluidization has been used to treat OPI to improve its functional properties including, solubility, foaming capacity, emulsifying activity, and water and oil holding capacity by mainly modifying the protein structural conformation of OPI. The improvement in functional properties was mainly attributed to the alterations in secondary structures with increasing α-helix, β-sheet, and β-turn structures and decreasing random coil as well as increase in the surface hydrophobicity, free sulfhydryl content and fluorescence quenching (Cheng et al., 2022). Another oat protein modification techniques with the use of additives was reported by Li & Xiong (2023), who suggests that the use of reducing agents to cleave disulfide bonds between acidic and basic subunits of 12S globulin can lead to improvements in surface active properties of OPI. Moreover, promising results were reported by using cysteine to breakdown the disulfide bonds of oat globulin which increased the emulsifying activity by 37 %. These results demonstrate the possibility of using OPI treated with reducing agents in food systems such as creams, salad dressing, and functional beverages (Li & Xiong, 2023). In a study that was carried out to investigate the effect of enzyme-aided ultrafiltration with and without deamidation on foaming properties of oat proteins it was found that enzyme-assisted extraction coupled with deamidation could improve the foaming properties of oat proteins (Immonen et al., 2022). Furthermore, the research was extended to study the effect of defatting on

foaming properties and reported that the removal of non- polar lipids could result in about 4–5 times higher foam volume and a stable foam compared to non-defatted OPI (Immonen et al., 2022). Furthermore, OPC has been treated with transglutaminase and used to develop meat like texturized products through high-moisture extrusion processing (Pöri et al., 2022). The study reported that transglutaminase treatment coupled with heat treatments was able to improve the cross-linking between proteins and enhance fibrous structure formation during extrusion. Novel food packaging films have also been developed using oat protein and the preservation effect on fresh commodities have been studied. According to Kang et al. (2023), the composite films made of oat proteins, pullulan, and nisin were able to preserve fresh strawberries revealing better bacteriostatic properties compared with the pure oat protein or pullulan films.

In summary, recent research has focused on optimizing extraction techniques to obtain OPI or OPC with enhanced functional properties, as well as treatments that can be performed on the extracted OPI or OPC for the same purpose. Given the versatility of oat protein and the novel research that has been conducted it is very likely that OPI or OPC could become a leading contender in the plant protein market, not only as a food ingredient but also in non-food applications such as eco-friendly packaging.

2.10. References

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Chapter 3: Determining the Impact of Genotype × Environment on Oat Protein Isolate Structural and Functional Characteristics

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3.1. Abstract

The effect of G × E on oat protein structural characteristics and functionality was studied using three oat genotypes grown in three different environments across the Canadian Prairies. The protein content of OPI ranged from 76.9% to 90.2 %. All structural characteristics except protein surface hydrophobicity were significantly impacted by G × E, whereas protein surface hydrophobicity strongly depended on the growing environment. SDS-PAGE and FT-IR analysis demonstrated oat protein profile and secondary structure were significantly impacted by genotype rather than environment. The cultivar Summit grown in Alberta had the highest foaming capacity which was significantly higher than Summit grown in the other environments, indicating a strong influence of environment on the foaming capacity. The structural-functional properties of OPI extracted from the given cultivars are influenced by G × E, however, further studies are needed to evaluate the stability of the tested cultivars over several years. Understanding how oat protein structure and functional properties are influenced by G × E will allow better utilization of OPI in the food industry by facilitating the development of OPI ingredients with predictable functional properties. **Keywords**: Oat protein isolate, Genotype × Environment, Structural characteristics, Functional properties

3.2. Introduction

Oat has received attention as a low-cost plant protein source with good nutritional value (Ma & Wood, 1987). Soy, pea, and wheat gluten are commonly used in plant protein product formulations and are currently available in the market. However, Oat protein is considered a promising alternative and a novel protein ingredient with good nutritional value and functionality for diverse applications (Mel & Malalgoda, 2021).

Oat contains approximately 12-20% protein and has four major protein fractions, 70%-80% globulin, 4%-15% prolamins, 1-12% albumins, and <10% glutelins (Nieto-Nieto et al., 2015). Furthermore, the protein composition of oat is considered to be unique in comparison to other cereals, including wheat, barley, and rye, due to high globulin protein (70-80%) content, as most cereals contain high levels of prolamins (Robert et al., 1983). The high globulin content is a driver for exploring oat as a non-GMO alternative to soy proteins. Oat proteins have commonly been extracted as either OPI or OPC. AE-IEP method (the wet milling process) is generally used to produce OPI (>90% protein) from defatted oat flour, whereas dry extraction methods produce OPC (60-70% protein), which have lower protein content (Yue, Gu, et al., 2021b).

From a nutrition perspective, oat contains more essential amino acids compared to other cereals and is referred to as a good quality plant protein ingredient (G. Liu et al., 2009). Apart from being a good source of protein, oat protein has important functional properties such as aqueous solubility, gelling properties, emulsifying properties, water and fat binding properties, and foaming ability (Yung Ma, 1983). As the protein's functional characteristics are influenced by its structure, it is crucial to understand the structure-function relationship of any protein for its use in the food industry. A previous study showed the possibility of using oat protein as a meat binder or extender due to the excellent fat binding and emulsification properties of oat proteins (Ma & Harwalkar,

1984). Considering the gelling properties of OPI, oat protein could form a heat-induced gel at neutral pH (Nieto-Nieto et al., 2014b). The authors also determined that the ability of oat protein to mimic thermal gelation of soy protein is associated with the structural similarity between soy glycinin protein and oat 12S globulin proteins.

Canada is the second-largest oat producer and the largest oat exporter worldwide. The Canadian Prairies, including Manitoba, Saskatchewan, and Alberta account for about 90% of oat production in Canada (Yan et al., 2011). The genetic makeup alone does not decide how crops perform in terms of yield, disease resistance and drought tolerance but also variations in environmental conditions, such as light radiation, precipitation and heat stress also introduce changes to how crops perform in fields (Buerstmayr et al., 2007). G × E occur in all crops and are important to plant breeders, agronomists, and food scientists, due to the confounding effects these interactions introduce to genotypes tested in different environments (Gullord & Aastveit, 1987). The grain attributes including yield, groat percentage, protein, and β-glucan content are influenced by G × E (Mut et al., 2018). A previous study reported that, oat protein and β-glucan content are equally influenced by genotype and environment while the lipid content is strongly influenced by genotype (Doehlert et al., 2001). As such, the influence on oat protein structure and functionality when grown in the Prairies is of great importance to develop oat protein ingredients with good functionality, thereby increasing the utilization of oat protein in the food industry.

The aim of this study was to evaluate the impact of G × E on oat protein structural and functional properties using three different genotypes grown in three locations across the Canadian Prairies. To our knowledge, effects of genotype, environment, and their interaction (G × E) on structural and functional properties of oat proteins has not been reported before.

3.3.Materials and methods

3.3.1. Materials

The experiment used grains collected from three oat cultivars (Summit, AC Morgan and CS Camden) grown at three different locations (Brandon, Manitoba, Saskatoon, Saskatchewan and Lacombe, Alberta) across Western Canada in 2020. At each location, the three cultivars were grown in trials with three replicates of each cultivar. A composite sample composed of all three replicates was created for each cultivar at each location and used for subsequent analysis.

3.3.2 Extraction of OPI

Oat grains were dehulled using a CODEMA dehuller (Minneapolis, MN, USA) and milled using a Retsch mill (ZM 200, Retsch GmbH & Co, Haan, Germany). The method for oat protein extraction was optimized upon testing different defatting and extraction conditions which could provide a good balance between protein content and yield. Flour was solubilized at pH 9.0 and 9.2 and the isoelectric precipitation was tested at pH 5.3, 5.5 and 5.7 while changing the number of extraction cycles from one to two and the method discussed below was selected based on the isolated protein content (82.2 %) and yield (69.6 %). In the finalized method, oat flour was first defatted using hexane in a 1:5 (flour : solvent) ratio using cold extraction method (Yung Ma, 1983). Defatted oat flour was then used to prepare OPI using a previously described method with modifications (Yung Ma, 1983). First 1:5 (flour: solvent) oat flour was dissolved in distilled water and pH was adjusted to pH 9.0 using 2M NaOH. After stirring at room temperature for 1 hour, the solution was centrifuged in 4000 g for 20 min at room temperature, the supernatant decanted, and protein was precipitated by adjusting the pH to 5.3. Centrifugation was performed again at 4000 g for 20 min at room temperature, and the resulting protein pellet was solubilized in water at pH 7.0 and freeze dried to obtain OPI. The protein content, starch and the moisture content were measured

through the micro Kjeldahl method (AACC method 46-13.01), Megazyme total starch assay (AACC method 76-13.01) and oven drying method (AOAC official method 930.15) respectively.

3.3.3. Structure Characterization of OPI

3.3.3.1. SDS- PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular weight of oat peptide subunits. Protein samples were mixed with sample buffer (Laemmli buffer for non-reducing conditions, and Leammli and β-mercaptoethanol for reducing conditions) and heated at 100 °C for 5 min and then cooled to room temperature, after which 5 µL of each sample were loaded to Criterion TGX Precast Polyacrylamide Gel, 4-20%, and subjected to electrophoresis at a constant voltage of 200 V for 35 min. After electrophoresis, the gel was stained with Coomassie Blue and detained overnight before scanning using a molecular imager (Gel Doc™ XR, Bio-Rad Laboratories, Hercules, CA) and analyzed through Image Lab™.

3.3.3.2. Surface hydrophobicity

Protein solutions of 0.05% (w/v) were prepared using 0.017M: 0.165M citric acid: sodium phosphate buffer solution and stirred for 2 hours at room temperature. Then a series of protein solutions ranging from 0.005 - 0.050% (w/v) concentration was prepared and 200µL of each solution was transferred to a 96 well plate to determine relative fluorescence index (RFI). The ANS fluorescence probe (Ammonium 8-Anilino-1-naphthalenesulfonate) was used with a microplate fluorescence reader (Bio-Tek FLx800,Winooski, VT, USA) to obtain RFI data. The relative fluorescence intensity was measured with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Surface hydrophobicity was expressed as the slope of the linear regression of fluorescence intensity versus protein concentration (Kato & Nakai, 1980).

3.3.3.3. Denaturation Characteristics

The denaturation characteristics of OPI were determined using a differential scanning calorimeter (Q200 Series™ DSC, TA Instruments, New Castle, DE, USA) (Yue, Zhu, et al., 2021). In brief, 10-15 µL of oat protein solutions (15.0 wt. %) were loaded into a Tzero hermetic pan and then hermetically sealed. The sealed pan was heated from 20 to 150℃ at a rate of 10 ℃/min using an empty pan as the reference. Onset temperature (T_o), end set temperature (T_E), and denaturation temperature (T_d) were determined, and data were analyzed using TA Universal Analysis software.

3.3.3.4. Fourier transform infrared spectroscopy (FT-IR) analysis

Freeze dried oat protein powders were analyzed using an INVENIO-R (Bruker Optics GmbH, Ettlingen, Germany) FT-IR spectrometer equipped with MIRacle^{TM} ATR (PIKE Technologies, Madison, WI, USA), according to a previously described method with modifications (G. Liu et al., 2009). A small amount of freeze dried OPI was placed on the ATR and pressed onto the diamond crystal surface tightly by the ATR accessory to ensure good contact with the ATR crystal. Eight independent scans were averaged for each sample proceeded by 32 scans for background for a spectral range of 400-4000 cm $^{-1}$. After spectral collection, ATR correction and background subtraction was carried out using the OMNIC software version 7.2a. The spectra were zoomed to analyze amide I and amide II bands (1800 - 900 cm $^{-1}$). The area percentage under amide I and amide II regions were compared in terms of genotype and environment to evaluate the impact of G×E on oat protein secondary structure.

3.3.4. Techno-functionality of OPI

3.3.4.1. Solubility of OPI

Solubility was measured using a Kjeldahl nitrogen analyzer (FOSS KT 200 Kjeltec, Hillerod, Denmark). OPI solutions (1%-5% (w/v%)) were prepared using distilled water and stirred for 2 h

to completely solubilize proteins. The pH was adjusted to 7.0 and centrifuged at 14196 g for 10 min and the supernatant was decanted. Then the protein content was analyzed using the Kjeldahl method and the solubility of protein in the supernatant was expressed using the following equation.

$$Solubility = \frac{protein\ content\ in\ the\ supernatent}{protein\ content\ in\ solution\ before\ centrifugation} * 100\ \%$$

3.3.4.2. Foaming properties

One g of OPI was first solubilized in 200 mL distilled water and pH was adjusted to 7.0 and stirred for 2 h. Then 50 mL of the prepared solution was foamed at 800-900 rpm (using Sunbeam Mixer at level 3) for 2 min and then transferred to a 250 mL graduated cylinder to measure the foam volume. Foaming capacity was expressed as the amount of foam (mL) produced per 1g of protein powder. The foam volume after 30 min was also recorded to calculate foam stability (Bera & Mukherjee, 1989).

$$Foam\ capacity = \frac{Initial\ foam\ volume}{g\ of\ protein\ in\ the\ solution}$$

$$Foam\ stability = \frac{Final\ foam\ volume}{Initial\ foam\ volume} * 100\ \%$$

3.3.4.3. Thermal gelation

Protein solutions of 20% (w/v %) were prepared in 0.1 N citrate acid buffer and pH was adjusted to pH 7.0 (Kong et al., 2008). Then 1 mL of each protein sample was heated in a water bath (95°C) for 20 min in microcentrifuge tubes. Protein gels were then completely cooled to room temperature before taking them out of microcentrifuge tubes. The OPI gels were analyzed using a texture analyzer (TA-XT Plus, Texture Technologies Corp., Hamilton, MA, USA) for maximum force need to rupture the gel.

3.3.4.4. Emulsifying properties

Emulsions were prepared by mixing 5 mL aqueous dispersions (containing 20 mg/mL protein in distilled water) of OPI with 1 mL of pure canola oil at pH 7.0 according to a previously described method (Osemwota et al., 2021). Then sample and oil mixtures were homogenized using the 10 mm shaft on a homogenizer (Fisher brand 850, Fisher Scientific, ON, Canada) at 20,000 rpm for 1 min. The droplet size ($d_{3,2}$) of the emulsions were analyzed by Mastersizer 2000 (Malvern Panalytical, Malvern, UK.) using distilled water as the dispersant. The emulsions were then transferred into a constantly sheared, ultrapure water (100mL) containing a wet sample dispersion component (Hydro 2000S) until about 13% obscuration level was achieved. All samples were measured in triplicates and the mean droplet size values were expressed as an indicator of emulsion capacity. The emulsion stability was analyzed by repeating the same method and determining the droplet size ($d_{3,2}$) 30 min after emulsion formation. Emulsion stability was expressed as a percentage ratio of the original value to the 30 min value.

3.3.3. Statistical analysis

Triplicate analyses were performed for each measurement. Statistical analysis was conducted as two-way ANOVA with genotype and environment as two factors to see the effect of genotype, environment, and G×E. Then, Tukey's pairwise comparisons for mean comparisons were carried out at $P < 0.05$ using the R software package (version 4.1.2).

3.4.Results and discussion

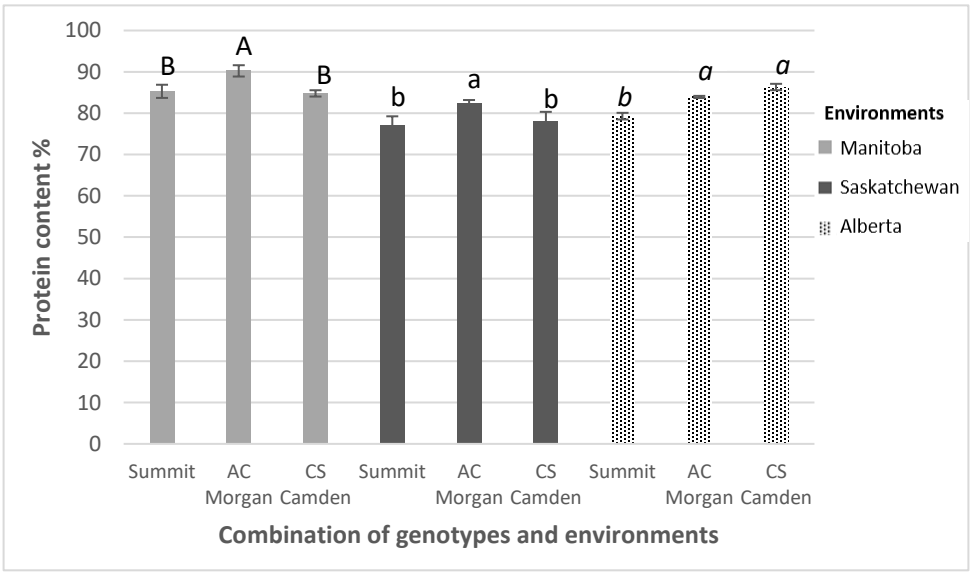
3.4.1. Proximate composition

The genotypes selected for this study are the most widely grown oat genotypes in the Canadian prairies (Canadian Grain Commision, 2022). The protein content of the OPI was determined (Figure 3.1.) through the micro kjeldahl method. In general, the numerically highest protein

content was seen in OPI from AC Morgan grown in Manitoba (90.2%) while the lowest protein content was recorded from the sample Summit grown in Saskatchewan (76.9%). This protein content is comparable with the protein content of soy and pea protein isolates having 93% and 84% protein content, respectively, when extracted through AE-IEP method (L'hocine et al., 2006). A significant effect of genotype, environment, and G × E was seen among OPI samples for protein content. Samples from AC Morgan grown in Manitoba had the highest protein content of 90.2 ± 1.4 % which was significantly higher than that of CS Camden and Summit from the same environment. Additionally, considering only the environmental impact, samples from Manitoba had significantly higher protein content than the other two environments. Therefore, the growing environment had a significant effect on the content of OPI obtained from the samples grown in the different regions. The protein content of the flour ranged from 12.7% to 15.8%. Another study reported that oat flour protein content is impacted by both genotype and environment, which is in agreement with the results of the present study as oat flour protein content is influenced by both genotype and environment but not by G × E (Doehlert et al., 2001). Flour samples from Saskatchewan had the highest protein content (15.1 %) while samples from Alberta had the lowest (13.4 %), and CS Camden had the highest flour protein content (14.8 %) while AC Morgan had the lowest (13.5 %) across genotypes. However, some studies have reported that the effect of the environment is much larger than that of genotype (Mut et al., 2018). The OPI protein extraction yield (% protein extracted based on flour protein content) was significantly impacted by growing environment and G × E, although genotype did not impact yield significantly. The highest protein yield was observed in the OPI samples from Alberta (53.8%) and Manitoba (48.2%), and also in terms of protein content, these two environments showed higher values compared to Saskatchewan. However, when comparing the protein content in the flour samples, flour from

Alberta had lower protein content (13.4 %) compared to samples from Saskatchewan (15.0 %) and Manitoba (14.0 %). Therefore, the protein extraction yield is not influenced by the protein content of starting flour as samples from Alberta had lower flour protein content while flour from Saskatchewan had the highest protein content as mentioned above. Additionally, the disparity between flour protein content and extraction yield may have an influence from the partial protein denaturation by defatting with hexane. Previous studies have reported that, defatting with hexane would lead to protein aggregation which might reduce the extractability during wet extraction process (Yue, Gu, et al., 2021b). The starch content of the OPI was very low and all samples contained ~0.2-0.5% starch. These results can be compared with another study by Liu et al. (2009) who reported a starch content of 1.0 % in OPI extracted with alkaline solutions. In relation to the data on OPI proximate analysis, the results indicate that OPI has 84-90% protein, <1% starch and 2-4% moisture.

(a)



(b)

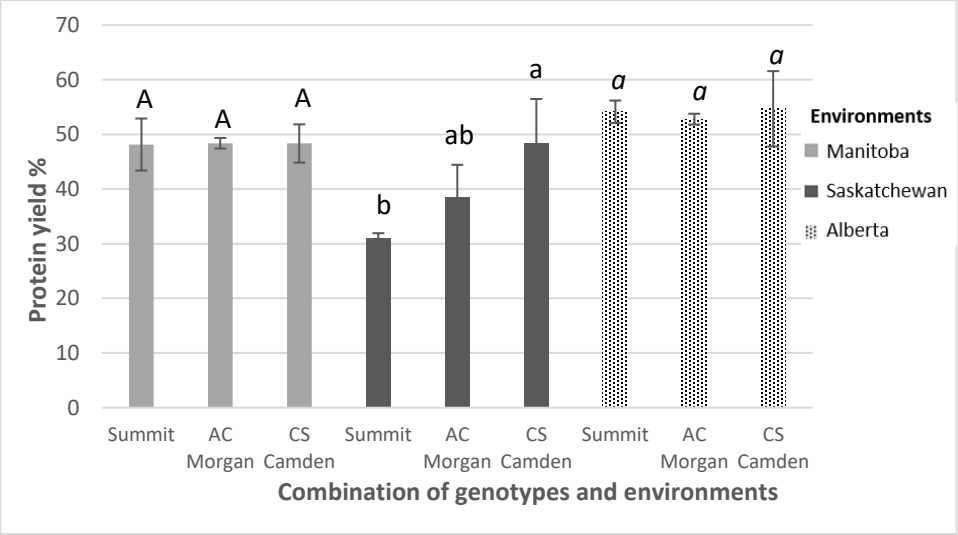


Figure 3.1. Protein content (a) and Protein yield based on flour protein (b) of oat protein isolate measured on three genotypes (Summit, AC Morgan and CS Camden) in each of three environments (Manitoba (MB), Saskatchewan (SK) and Alberta (AB)).

Mean value with different letters (A,a,*a*) indicates a significant difference at the $P < 0.05$ level within an environment.

3.4.2. Structure Characterization of OPI

3.4.2.1. SDS- PAGE

Figure 3.2. presents the SDS-PAGE for reduced and non-reduced samples of the three cultivars and three locations in Canada. The intense bands with protein molecular weights ranging from 14 kDa to 65 kDa were observed in both reduced and non-reduced samples. All OPI samples with reducing agents exhibited two major bands at ~36 kDa and ~22 kDa, corresponding to acidic (α) and basic (β) subunits of oat 12S globulin fractions, respectively. According to Peterson (1978), OPI extracted through AE-IEP methods contained ~80% globulin proteins. The non-reduced samples in the gel indicated six intense bands with molecular weights (MW) of ~66.2 kDa, ~57 kDa, ~45 kDa, ~36.0 kDa, ~23.0 kDa and ~14.4 kDa which were also reported in previous

studies (G. Liu et al., 2009). The authors also reported that the bands around 14 kDa could be a mixture of albumins and prolamins as both oat albumin and prolamins showed bands around 14-15 kDa (G. Liu et al., 2009). A previous study reported that oat prolamin fraction shows an intense band at 23 kDa which can also be seen in the non-reduced gel in this study (Figure. 2a) (Ma & Harwalkar, 1984). The authors reported that oat glutelins show bands at 18 kDa and 10 kDa, however these bands are not very prominent in the current study which could be due to the low proportion of glutelins in OPI. The intensity of bands between the molecular weight of 22 kDa and 37 kDa in the reduced gel was different among the cultivars from the same environment, which could be due to the strong genotypic influence on protein profile. The electrophoretic profile of OPI within the same genotype was very similar in both non-reduced and reduced conditions across the three environments. Given its similarity across environments, electrophoretic profile of oat proteins was strongly dependent on the genotype rather than the environment. The same relationship was also observed with regard to protein content of OPI even though there was a significant G×E interaction.

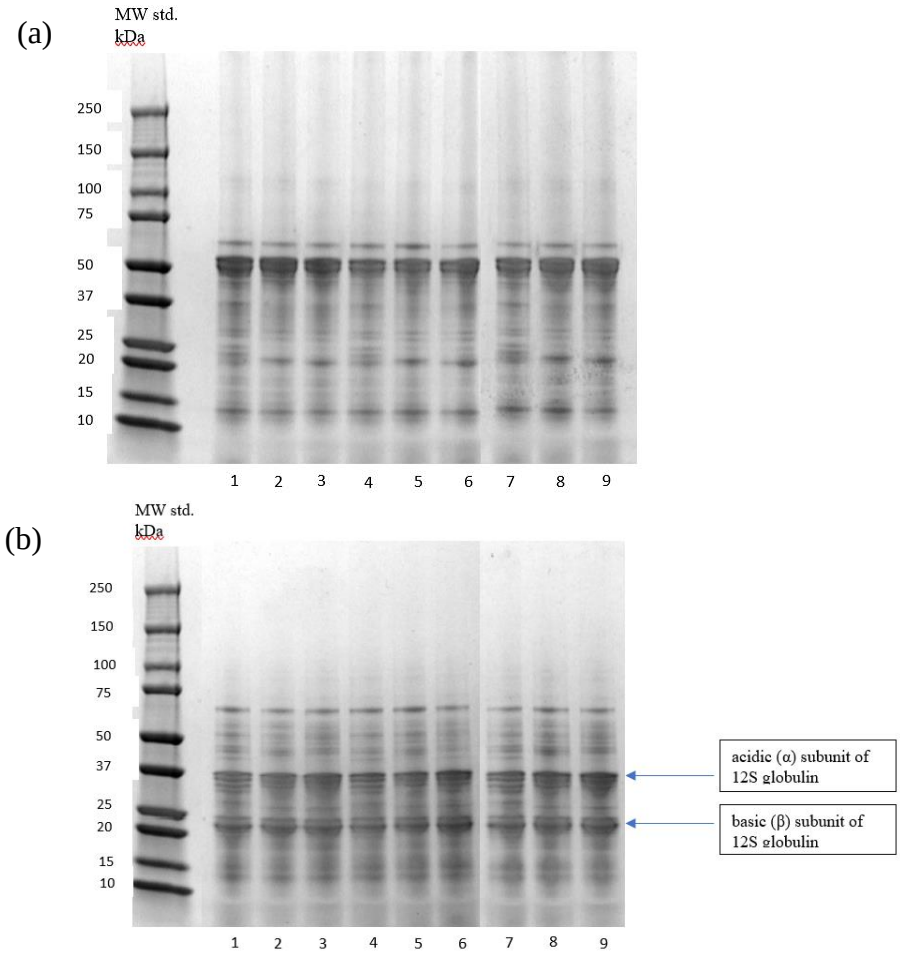


Figure 3.2. SDS PAGE of oat protein isolates in non-reduced (a) and reduced (b) conditions for the OPI samples

1 - Manitoba Summit 6 - Sask. CS Camden

2- Manitoba AC Morgan 7 - Alberta Summit

3-Manitoba CS Camden 8 - Alberta AC Morgan

4- Sask. Summit 9 - Alberta CS Camden

5- Sask. AC Morgan

3.4.2.2. Surface hydrophobicity

Usually, more hydrophilic residues are located on the surface while hydrophobic residues are buried in the center of the protein molecule (Nakai, 1983). According to previous studies on

structural characteristics of proteins, surface hydrophobicity determines its aqueous solubility and thereby influences all other functional properties (Bigelow, 1967). As an example, according to the Osborne classification of proteins, the surface hydrophobicity of gliadin and glutelins are higher than that of albumins and globulins (Jing et al., 2016). In this study, significant changes in surface hydrophobicity were found only among the three environments (Manitoba, Saskatchewan, and Alberta), but not among the different genotypes studied. Therefore, the results suggest that OPI surface charge is more dependent on the environment rather than genotype. According to a previous study on surface active properties of proteins and their association with structural characteristics, growing environment was identified as a major factor determining protein hydrophobicity (Konak et al., 2014). In the present study, samples from Alberta (Figure 3.3.) showed the highest surface hydrophobicity and Manitoba showed the lowest. As environment has a significant influence on surface hydrophobicity, these results are expected to change based on the changes in environmental conditions, such as drought, rainfall, fertilizer application and soil nutrients, disease conditions and temperature. Furthermore, the 2020 growing season was not affected by drought as much as the 2021 growing season, during which severe drought conditions were experienced across the Prairies, as such these observations could vary for samples from different growing years/environments (Government of Canada, 2021). Generally, elevated surface hydrophobicity is considered an indication of enhanced protein foaming capacity, as it shows the potential to interact with the air phase, however, hydrophobicity is also associated with bitter taste (Were et al., 1997).

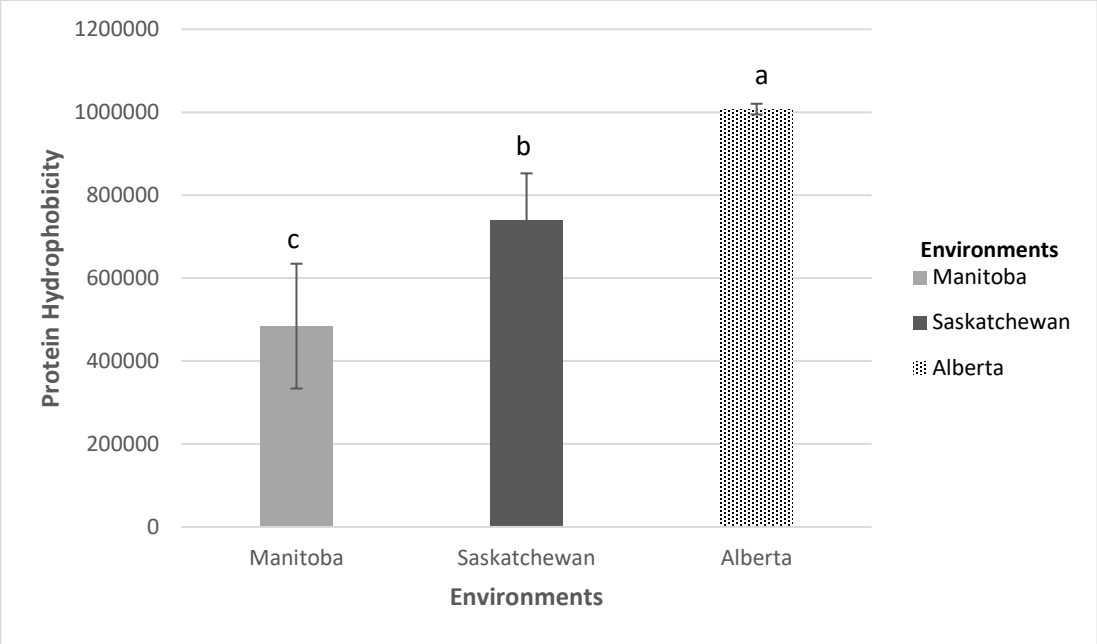


Figure 3.3. Surface hydrophobicity of oat protein isolates measured in three environments (Manitoba, Saskatchewan, and Alberta).

Mean value with different letters indicates a significant difference at the $P < 0.05$ level.

3.4.2.3. Denaturation Characteristics

The denaturation characteristics of proteins are of high importance for new product development in food and edible packaging applications. Peak denaturation temperature and enthalpy of OPI were both affected by oat genotype and environment. Peak denaturation temperature is an indicator of thermal stability of proteins, while the enthalpy is representative of the quantity of ordered secondary structure of a protein (Ma & Harwalkar, 1988). Although significant changes among genotypes and environment were detected in statistical analysis, in general the mean denaturation temperature of all OPI samples ranged from 110.2 °C to 111.6 °C (Figure 3.4.). According to Ma & Harwalkar (1984), the globulin fraction of oat protein has a sharp symmetrical endothermic peak with a denaturation temperature of 110 °C. These values are also in agreement with recent studies

suggesting that OPI has the similar denaturation temperature to that of the oat globulin fraction (Sunilkumar & Tareke, 2019;Yue, Zhu, et al., 2021). These results indicate that oat proteins are more thermally stable than most other plant proteins such as soy, hemp and pea, which show denaturation temperatures of 93.0 °C, 95.0 °C and 83.8 °C, respectively (Shevkani et al., 2015;(Tang et al., 2006). The ability of a protein to stay solubilized at elevated temperatures is essential for its application in protein beverage products which undergo pasteurization and other thermal treatments. The results of this study suggest that OPI could be considered as a good candidate for thermally processed food systems, where the structural integrity, hence functionality of OPI is preserved upon processing. In general the average enthalpy of OPI samples in this study ranged from 0.69 to 1.53 J/g, similar to the values (0.98 J/g) reported in previous work (Nieto-Nieto et al., 2014b). A significant effect of genotype, environment, and G × E was detected in terms of enthalpy among the samples, as shown in Figure 3.5. AC Morgan and CS Camden had a significantly higher denaturation enthalpy than that of Summit. Samples from Alberta and Manitoba had a significantly higher denaturation enthalpy than those from Saskatchewan. Furthermore, considering the G × E interaction, only samples from Saskatchewan showed significant differences among the three genotypes (Figure 3.5.). The impact from genotype could be related to variations in amino acid composition among the different genotypes. During crop development, environmental conditions during the different phases of grain filling could impact the overall protein composition. Therefore, environmental conditions can impact the deposition of these proteins, resulting in changes in protein composition of grains upon physiological maturity. The differences in the enthalpy could be associated with structural characteristics, such as protein hydrophobicity. During protein denaturation the native protein unfolds and exposes the buried interior of protein, resulting in more protein-protein interactions due to hydrophobic associations

(Ma & Harwalkar, 1988). As the disruption of hydrophobic interactions and protein aggregations are exothermic reactions, the differences in protein hydrophobicity among the OPI samples could reduce the total endothermic contribution from protein denaturation (Jackson & Brandts, 1970).

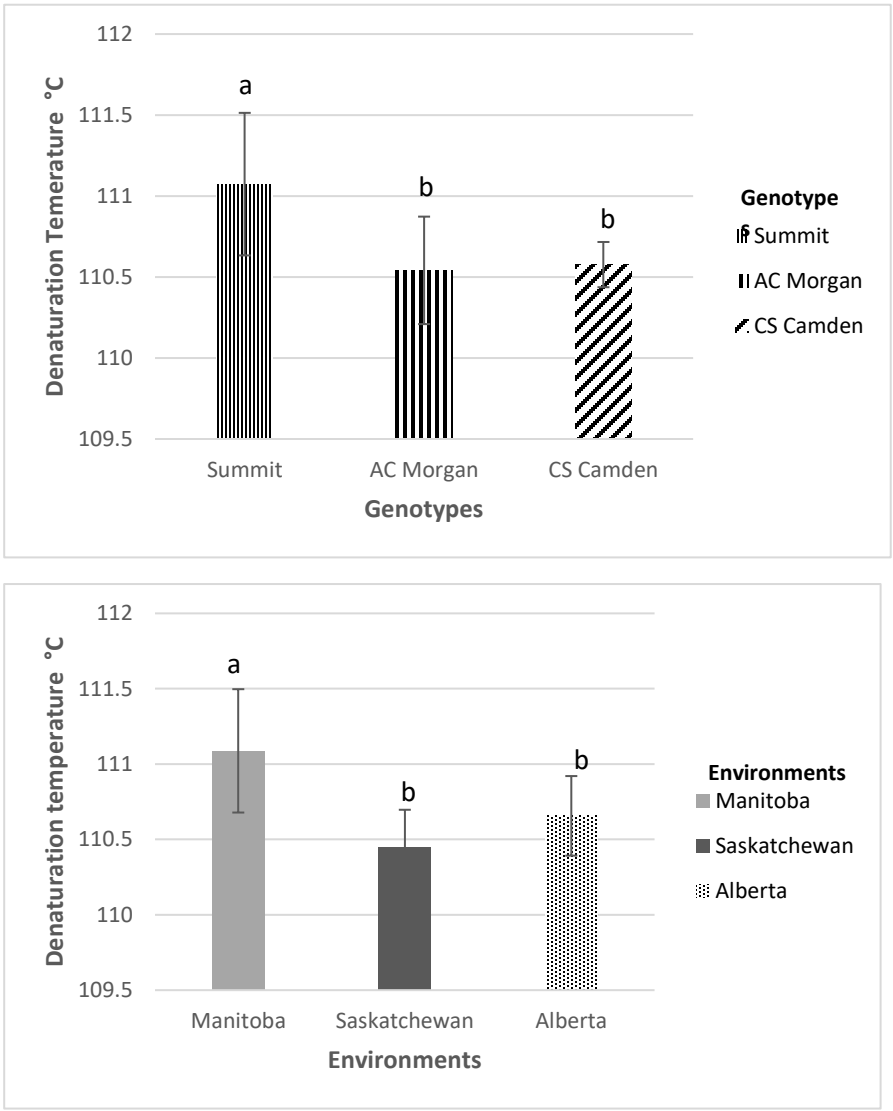


Figure 3.4. Denaturation temperature of oat protein isolates measured on three genotypes (Summit, AC Morgan and CS Camden) and in three environments (Manitoba, Saskatchewan and Alberta).

Mean value with different letters indicates a significant difference at the $P < 0.05$ level.

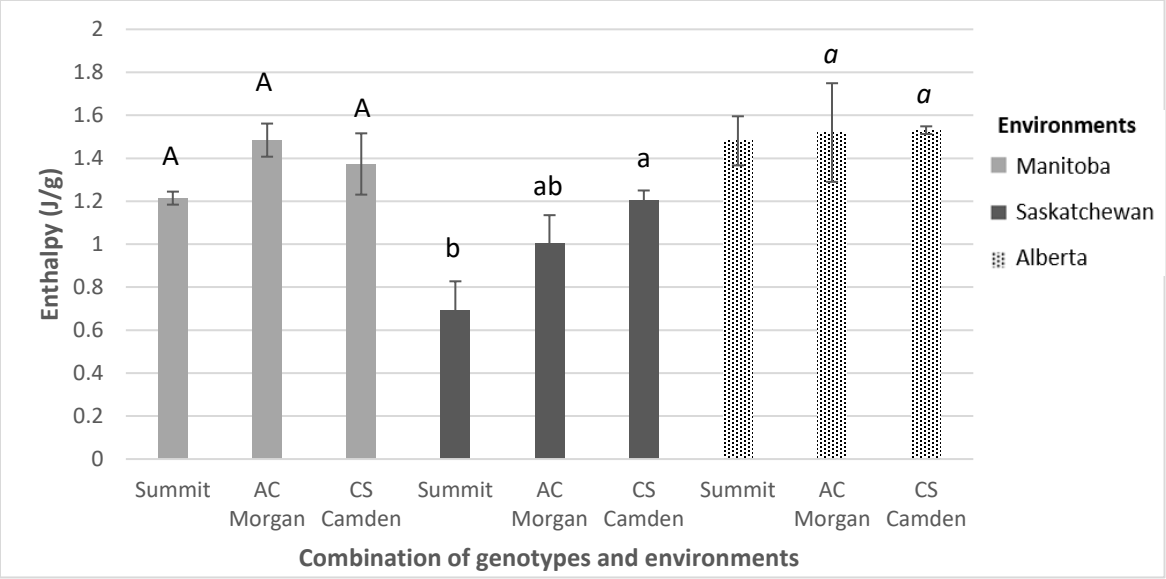


Figure 3.5. Denaturation enthalpy of oat protein isolates measured on three genotypes (Summit, AC Morgan and CS Camden) in each of three environments (Manitoba, Saskatchewan and Alberta).

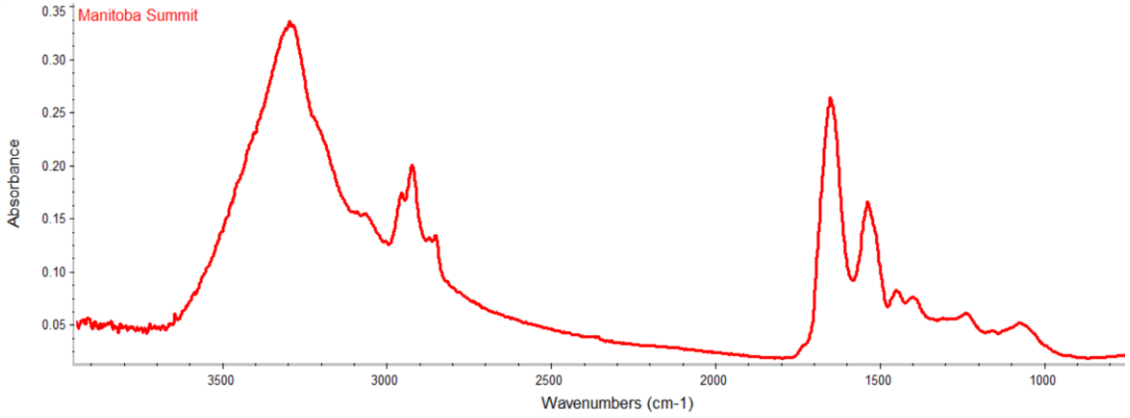
Mean value with different letters (A,a,*a*) indicates a significant difference at the $P < 0.05$ level within an environment.

3.4.2.4. Fourier Transform Infrared Spectroscopy

Figure 3.6. shows the FTIR spectrum of OPI. The amide I and amide II are located at approximately 1600-1700 cm $^{-1}$ and 1500-1600 cm $^{-1}$, respectively (Jing et al., 2016). The amide I is considered to be a combination of the CO stretch, CN stretch and C$^{\alpha}$CN deformation, while the amide II is associated with NH in-plane bend with a CN stretch, as well as a C$^{\alpha}$C stretch and some minor contributions (Hopkins et al., 1991). Furthermore, the peaks near ~3300 cm^{-1} and 2930 cm^{-1} are associated with the N-H stretching band and alkyl group stretching bands, respectively, according to previous work (G. Liu et al., 2009). Most previous studies have only focused on the amide I region and the second derivative has been used to estimate the secondary structure of proteins (Yue, Zhu, et al., 2021). Self-deconvolution analysis has been used to identify

component peaks within the broad amide I region. However, the accuracy of the results is still doubtful as these techniques are very subjective and challenging to reproduce. In the present study the FTIR spectra were analyzed as a comparison using both amide I and amide II area percentages to evaluate how genotype and environment impacts protein secondary structure. In statistical context, genotype, environment, and G × E interaction had no significant ($p > 0.05$) effect on the area percentage of amide I and amide II, suggesting that the protein characteristics defined by amide I and amide II are not dependent on genotype, environment or G×E interaction. However, in terms of genotype, the probability values were 0.05 and 0.06, for amide I and amide II, respectively, suggesting there may be an effect from the genotype even though it was not statistically significant in the present study. Additionally, the electrophoretic profile of oat proteins was also strongly influenced by the genotype rather than the environment, which is in agreement with the FTIR analysis results. Given its protein structural results, it can be concluded that both oat protein profile and secondary structure are impacted by oat genotype.

(a)



(b)

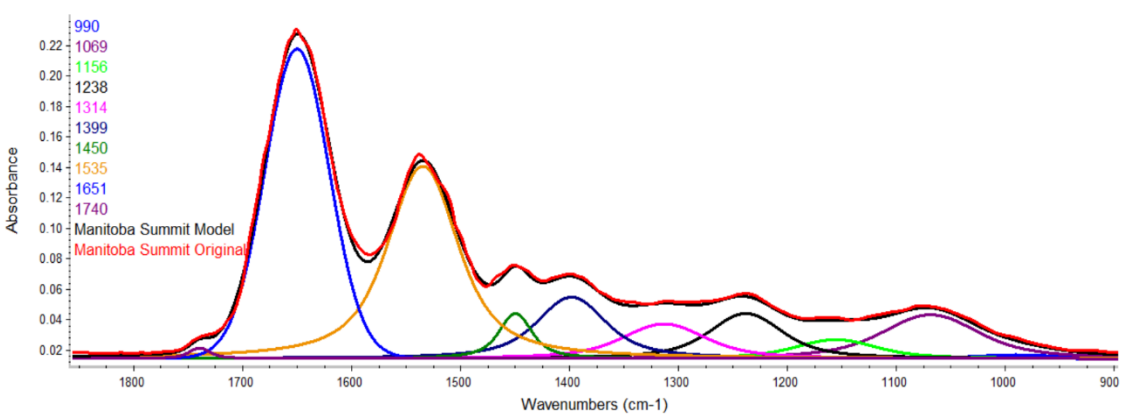


Figure 3.6. (a) FTIR spectrum of oat protein isolate (OPI), (b) Zoomed Amide I and II bands with fitted peaks measured from sample Summit in Manitoba.

3.4.3. Techno-functionality of OPI

3.4.3.1. Solubility of OPI

The solubility of OPI investigated at pH 7.0 is presented in Figure 3.7. The solubility profiles of OPI did not indicate significant differences for genotype or environment, however significant differences were found for G × E at $P < 0.05$. Generally, the solubility of OPI ranged from 13-33 % as determined by the micro Kjeldahl protein quantitation method. These results are in agreement

with other studies, which also reported low solubility values for OPI at neutral pH. Furthermore, previous studies reported that the oat globulin fraction had minimum solubility at pH 6-7 and the highest solubility at acidic and alkaline ends of the solubility curve (Ma & Harwalkar, 1984). According to Nieto-Nieto et al. (2015), OPI is comprised of ~80% globulin, which is likely why OPI in this study showed low solubility. In Alberta and Saskatchewan, Summit showed the highest solubility, however in Manitoba CS Camden showed elevated solubility in comparison to the other two genotypes. Aqueous solubility of a protein depends on the balance between hydrophilicity and hydrophobicity, which in turn depends on the amino acid compositions on the exposed surface of the protein molecule. As higher levels of hydrophobicity on protein surface could lead to reduced water solubility, the amino acid compositional changes in OPI across genotypes and environments could cause differences in water solubility as observed in this study. Even though samples from Alberta had the highest surface hydrophobicity, a reduced solubility could not be seen in the current study. Therefore, a relationship between the surface hydrophobicity of proteins and the solubility was not observed in this study, however as noted above, protein solubility is not determined by surface hydrophobicity alone.

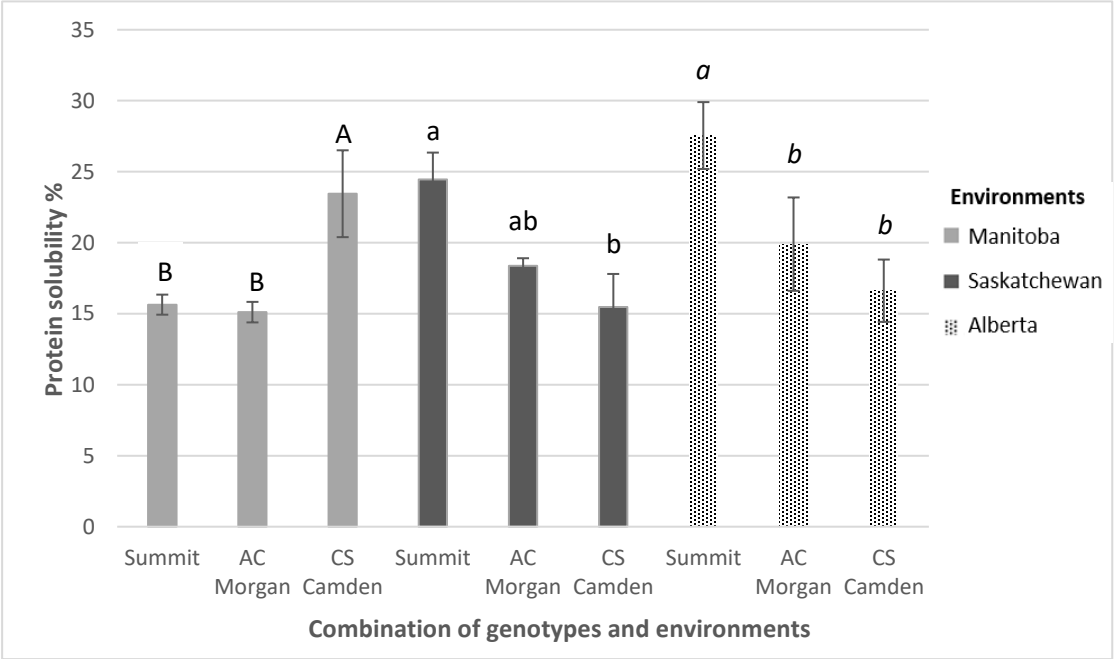


Figure 3.7. Protein Solubility % of oat protein isolate samples measured on three genotypes (Summit, AC Morgan and CS Camden) in each of three environments (Manitoba, Saskatchewan and Alberta).

Mean value with different letters indicates (A,a,*a*) a significant difference at the $P < 0.05$ level within an environment.

3.4.3.2. Foaming properties

Foaming capacity and stability of OPI are shown in Figure 3.8. and 3.9. Significant effects of genotype, environment, and G × E interaction were seen among OPI samples for foaming capacity, however only genotype and environment had a significant effect on foaming stability. The highest foaming capacity was observed in the samples from Alberta. Were et al. (1997) reported that elevated surface hydrophobicity of proteins has a close relevance to enhanced foaming capacity, therefore the increased surface hydrophobicity in samples from Alberta could have led to enhanced foaming capacity. A good foaming agent must contain both hydrophobic and hydrophilic groups as hydrophobicity and viscosity play an essential part in foaming capacity, whereas foaming stability depends on net charge (Nakai, 1983). Even though protein water solubility impacts

foaming capacity, the OPI in the current study had poor water solubility as noted above. Previous studies report the importance of the balance between electrostatic repulsions and hydrophobic interactions to balance protein-protein and protein-solvent interactions (Aluko & Yada, 1995). Interestingly, a relationship was found between hydrophobicity and elevated foaming capacity in the OPI from Alberta. Moreover, Summit and AC Morgan from Alberta had higher foam volumes than those grown in the other two locations indicating a strong effect of environment on the protein structure and functionality in terms of foaming capacity. However, considering the three genotypes from all three locations, Summit and AC Morgan had a distinguishable foam volume indicating higher foaming capacity than CS Camden, which suggests a genotypic effect on foaming capacity. In general, it can be stated that Summit and AC Morgan possess better foaming properties when grown in Alberta than the other two locations studied. The results of foaming stability followed the same pattern as of foaming capacity, however samples from both Alberta and Saskatchewan had elevated foaming stability compared to Manitoba. Both foaming capacity and stability showed differences among the genotypes in a similar pattern, with Summit ≥ AC Morgan > CS Camden. Even though a G×E interaction for foaming stability was not observed, samples from Alberta having better foaming stability showed that growing environment had a strong effect on OPI foaming capacity and foaming stability. High foaming stability of OPI indicates a strong interfacial film of the adsorbed proteins, and all OPI samples having foaming stability higher than 70% demonstrate the possibility of OPI to be a good foaming agent. The use of hexane in defatting treatment can exert adverse effects on the functionality of OPI. According to previous work, OPI extracted from defatted flour exhibited excellent foaming capacity compared to proteins extracted from non-defatted flour however, in terms of foaming stability, proteins extracted from non-defatted flour showed better foaming stability compared to proteins from defatted flour (Yue, Gu,

et al., 2021b). The reduced foaming stability of proteins extracted with defatted flour was mainly due to their partial denaturation with hexane which reduces the protein-protein interactions to form the rigid interfacial film (Yue, Gu, et al., 2021b). Furthermore, another study reported that defatting oat flour using supercritical carbon dioxide (SC-CO_2) could increase the soluble protein content compared to methods involving organic solvents, by selectively removing non-polar lipids from oat flour without causing any protein denaturation (Konak et al., 2014). However, the effect of defatting treatment on the functional properties were not analyzed in this study as all OPI were extracted from defatted oat flour using hexane.

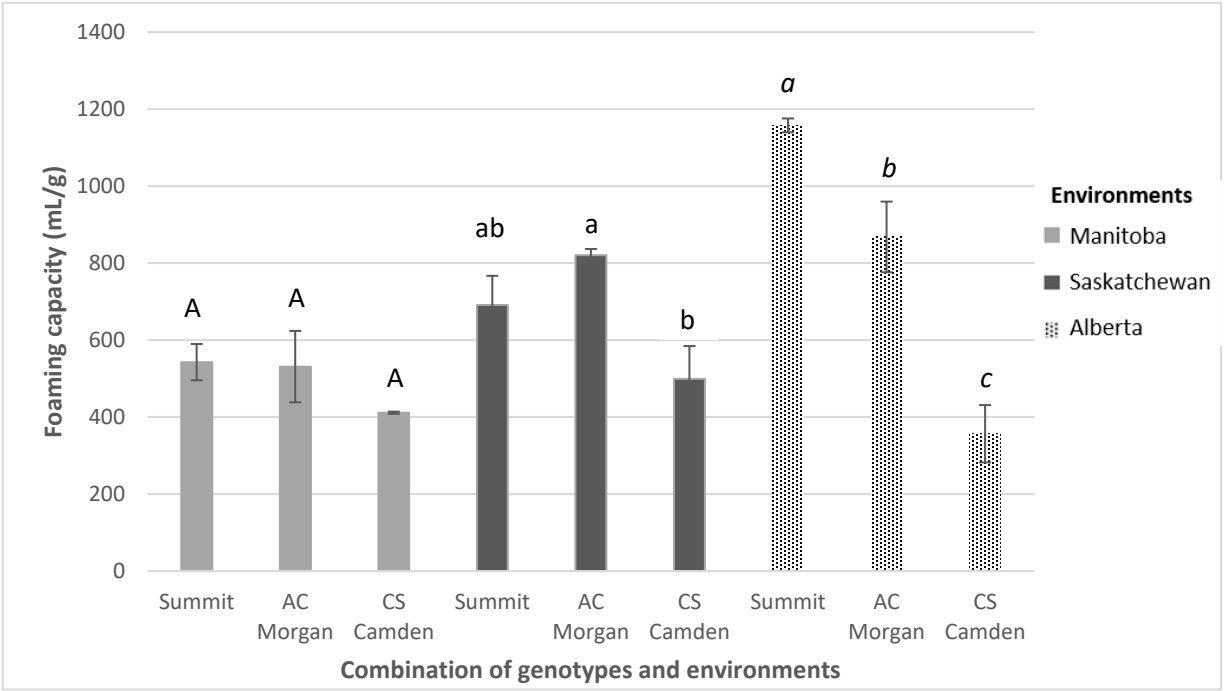
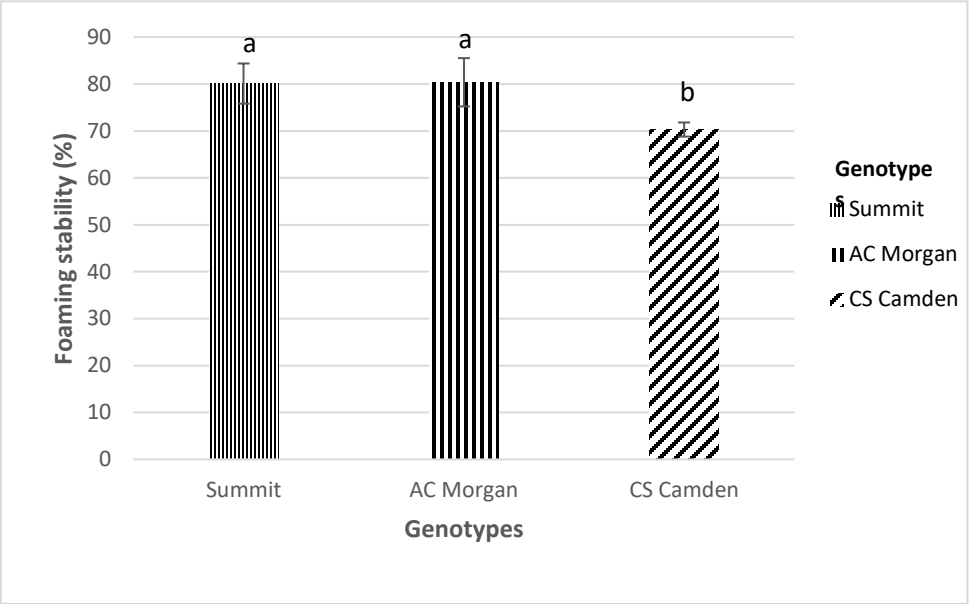


Figure 3.8. Foaming capacity (mL/g) of oat protein isolate samples measured on three genotypes (Summit, AC Morgan and CS Camden) in each of three environments (Manitoba, Saskatchewan and Alberta).

Mean value with different letters (A,a,*a*) indicates a significant difference at the $P < 0.05$ level within an environment.



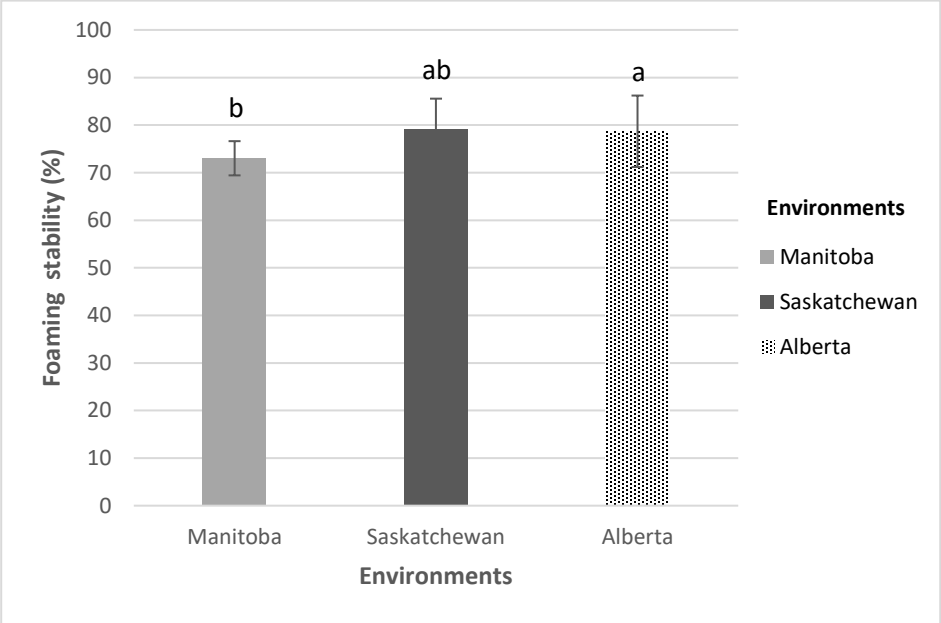


Figure 3.9. Foaming stability % of oat protein isolate samples measured on three genotypes (Summit, AC Morgan and CS Camden) and in three environments (Manitoba, Saskatchewan and Alberta).

Mean value with different letters indicates a significant difference at the $P < 0.05$ level.

3.4.3.3. Thermal gelation

The differences in the hardness (force required to attain full deformation) of OPI gels are shown in Figure 3.10. All samples formed soft gels with minor visible differences among them. Similar observations were made by Ma & Wood (1987), who reported that gels made of native OPI tend to be weak at both pH 8.5 and 9.5, while Ma & Khanzada (1987) reported that 10% OPI was unable to produce a gel at pH 7.5 and formed a weak gel at pH 9.7. The low strength and gelling ability are mainly due to low solubility observed in all OPI samples. The effect of environment on gel hardness was not seen among all samples, however genotype and G × E significantly impacted gelling properties. Considering the genotypic effect on gel strength, the order was found as, CS Camden ≥ AC Morgan ≥ Summit for samples from Manitoba and Alberta but not in Saskatchewan, which showed no significant differences among the genotypes within the three environment. CS Camden and AC Morgan had elevated hardness compared to that of Summit, even though not statistically significant across all three environments. OPI produced weak gels compared to soy protein isolate even though both contain similar proportions of globulin proteins as the major protein component. A protein must undergo a certain amount of denaturation before a gel can be formed (Renkema & Van Vliet, 2002). The OPI in this study displayed a denaturation temperature around 110-111℃ whereas the major soy protein components, β-conglycinin and glycinin have their denaturation temperatures at 70 °C and 80 °C, respectively. Therefore, the differences in denaturation temperature can lead to differences in the gelling properties as the gel formation was performed between 90-95°C. Given that the denaturation temperature is higher than the temperature used for gelling, perhaps OPI samples did not undergo sufficient denaturation to form a good gel.

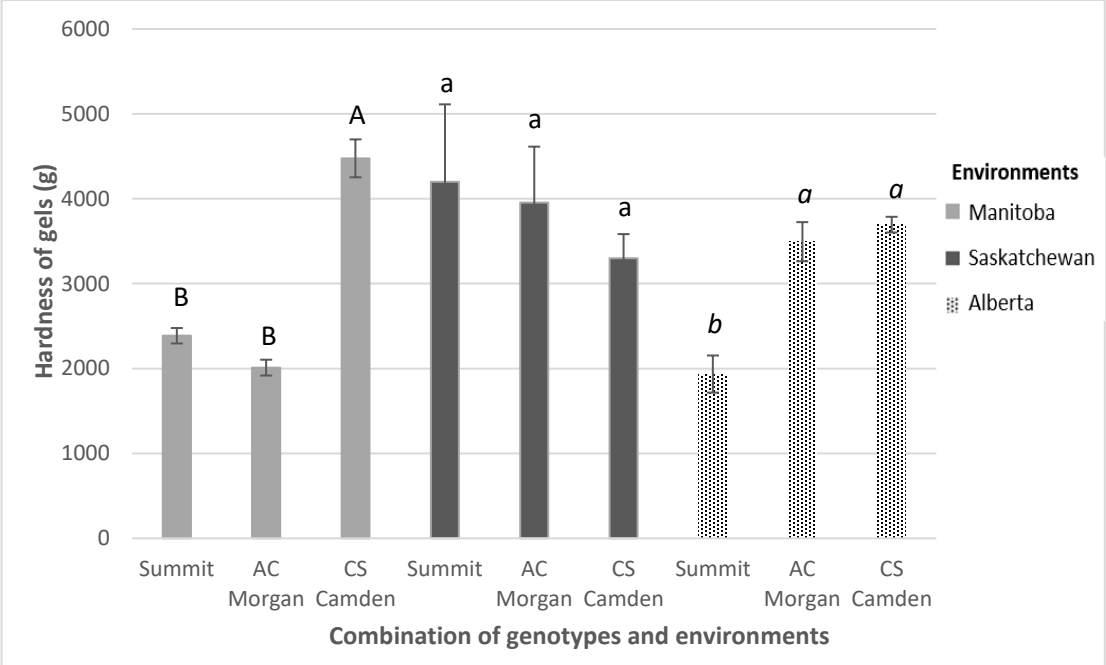


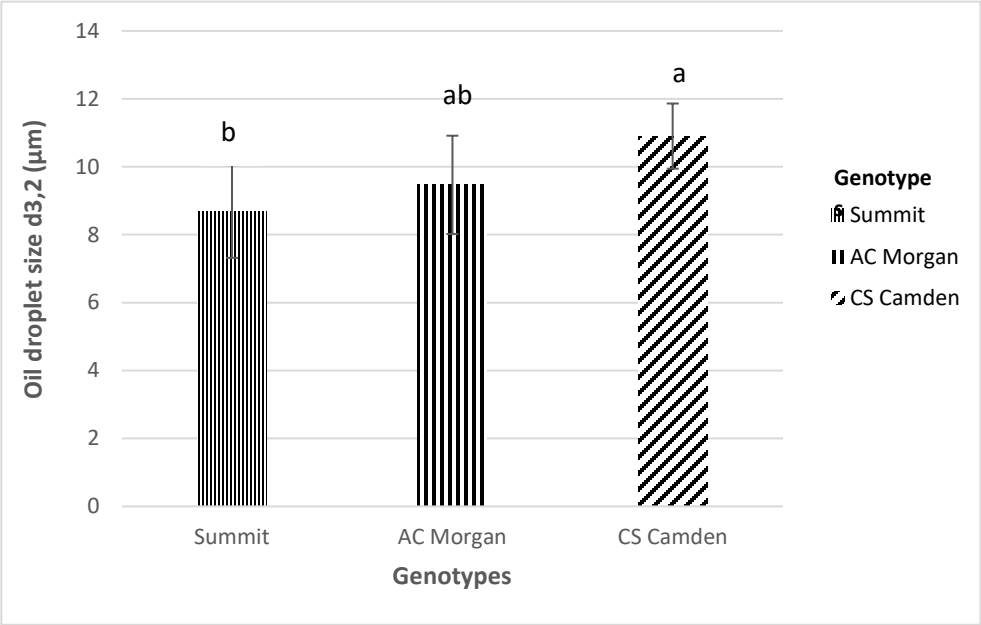
Figure 3.10. Gelling capacity (Max force to rupture gel) (g) of oat protein isolate samples measured on three genotypes (Summit, AC Morgan and CS Camden) in each of three environments (Manitoba (MB), Saskatchewan (SK) and Alberta (AB)).

Mean value with different letters (A,a,*a*) indicates a significant difference at the $P < 0.05$ level within an environment

3.4.3.4. Emulsion Formation and Stability

In emulsions, proteins migrate to the interface between oil and water to form a thin layer around the oil droplets orienting its hydrophilic groups towards water and the hydrophobic groups towards the oil phase to reduce the interfacial tension (Graham & Phillips, 1976). Therefore, emulsion capacity of a protein is dependent on the balance between the hydrophilicity and hydrophobicity of proteins. Changes in the droplet size and stability of the emulsion formed using OPI are shown in Figure 3.11. and 3.12. The droplet size is an indicator of emulsion capacity. A significant effect from both genotype and environment was found among OPI in terms of emulsion capacity. Samples from Saskatchewan produced emulsions with the smallest mean droplet size which was

8.7 ±1.7 µm in diameter in terms of environment. The Alberta samples, which had elevated surface hydrophobicity, did not show any significantly improved emulsion capacity compared to the other two environments. Therefore, other factors such as protein composition or structure may be more influential for emulsion capacity than surface hydrophobicity. The genotype Summit had the smallest droplet size 8.7 ±1.3 µm. However, G × E interaction was not found among samples in relation to emulsion capacity. Emulsion stability is indicative of how long an emulsion can be kept unchanged under specific conditions (Kinsella, 1976). All emulsions were very stable after 30 min and had no visible layer separation even after 2 hours. Growing environment and G × E ($P < 0.05$) significantly impacted emulsion stability of OPI, even though the genotype did not show a significant impact. The highest emulsion stability was recorded in the samples from Manitoba and the lowest in those from Alberta. However, significant differences among genotypes were only observed for the samples taken from Saskatchewan where the highest emulsion stability was shown by Summit (76.4%), however a similar pattern was not observed in the other two environments. Even though the smaller oil droplet size is known to be associated with enhanced emulsion stability, such a relationship was not seen in the current study (Osemwota et al., 2021). The stability of protein emulsions in this study can be explained by the phenomenon of having strong repulsive forces away from the isoelectric point of OPI (pH 5.3), as the emulsions were prepared at pH 7.0. Furthermore, all three genotypes from Manitoba showed the highest emulsion stability and explain the strong effect of growing environment on emulsion stability. Again, as these results are dependent on the conditions (temperature, pH, etc.) used, the emulsion stability results can vary when tested under different conditions.



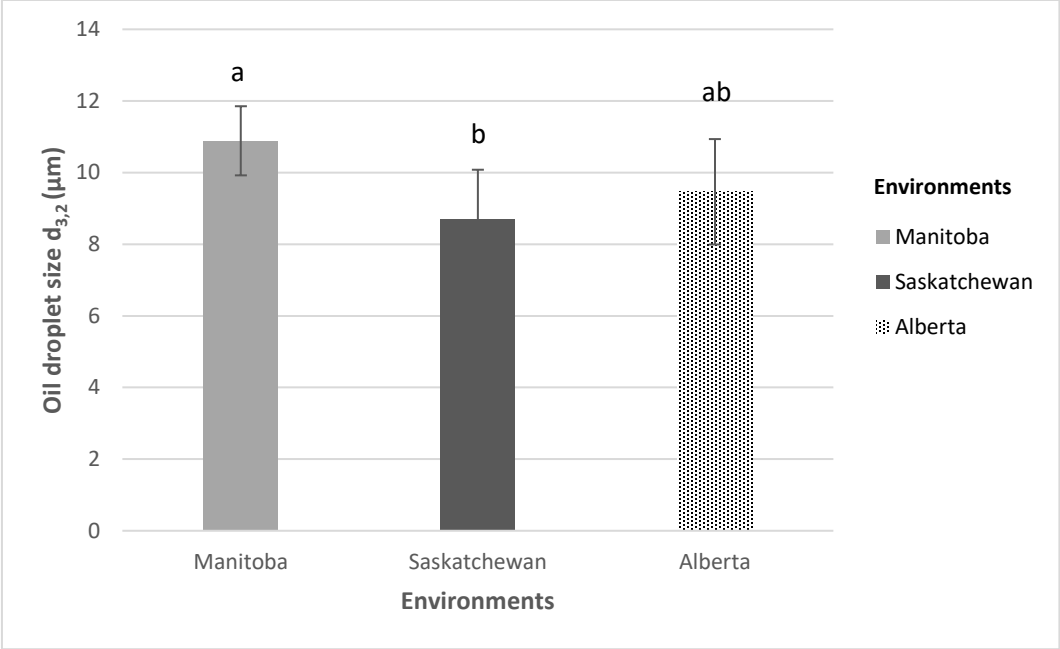


Figure 3.11. Oil droplet size d3,2 (µm) of oat protein isolate samples measured on three genotypes (Summit, AC Morgan and CS Camden) and in three environments (Manitoba, Saskatchewan and Alberta).

Mean value with different letters indicates a significant difference at the $P < 0.05$ level.

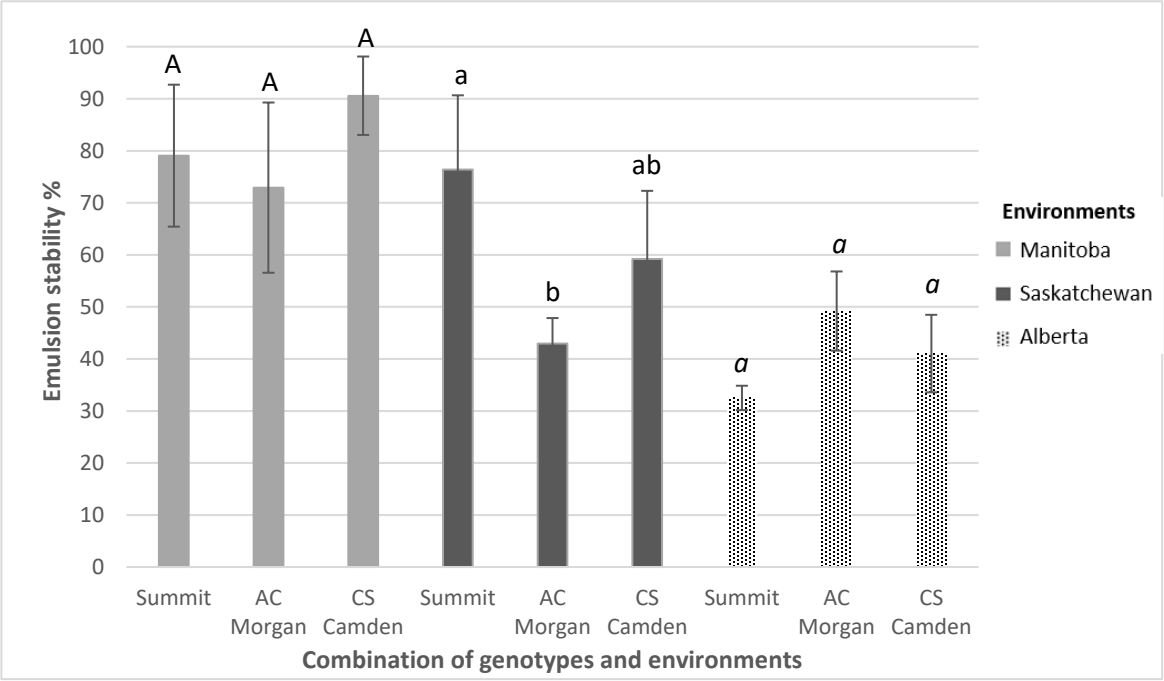


Figure 3.12. Emulsion stability % of oat protein isolate samples measured on three genotypes (Summit, AC Morgan and CS Camden) in each of three environments (Manitoba, Saskatchewan and Alberta).Mean value with different letters (A,a,*a*) indicates a significant difference at the $P < 0.05$ level within an environment.

3.5. Conclusion

Oat protein quality is governed by both nutritional aspects and functional properties that are useful for various downstream industrial applications. Oat protein content and quality traits are affected by genotype and response to environmental conditions. In particular, the consistency in OPI is of great importance to the food industry as a food ingredient regardless of the influence of the environment. The current study investigated G × E interaction on structural and functional properties of oat proteins using three popular oat genotypes widely grown across the Canadian prairies. Our study showed that oat protein content, protein profile, and functional properties are dependent on genotype and the environment. The results indicate that G × E significantly impacts

OPI structure and functionality including denaturation enthalpy, protein solubility, foaming capacity, emulsion stability, and textural characteristics. This study was based on OPI extracted from grain obtained from the 2020 growing season, however the structural and functional properties could vary across different years (i.e., environmental conditions). This study did not focus on the correlation between structural and functional properties. The environment significantly impacts all the OPI characteristics measured, highlighting the need for further research to assess oat cultivars grown at multiple locations over several years to identify the most suitable cultivars and growing environments for targeted applications in the food industry.

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Chapter 4. Changes in oat protein composition in response to variations in genotype and environment determined through SE-HPLC and LC-MS

4.1. Abstract

The effect of genotype and environment on oat seed protein composition was analyzed through SE-HPLC and LC-MS to characterize proteins in OPI extracted from three genotypes grown at three locations on the Canadian prairies. SE-HPLC exhibited 4 fractions for OPI including polymeric globulins, avenins, glutelins and albumins and smaller proteins. The protein composition was dependent on the environment rather than the genotype. The proteins identified through LC-MS were grouped into 8 categories including globulins, prolamins/avenins, glutelins, enzymes/ albumins, enzyme inhibitors, heat shock proteins, grain softness proteins and allergenic proteins. Three main globulin protein types were also identified including P14812|SSG2-12S seed storage globulin, Q6UJY8_TRITU-globulin and M7ZQM3_TRIUA-Globulin-1 S allele. PCA analysis indicated that samples from Manitoba show a positive association with M7ZQM3_TRIUA-Globulin-1 S allele and Q6UJY8_TRITU-globulin while samples from Alberta and Saskatchewan had a negative association. The results show that the influence of G×E on oat protein fractions and their relative composition is crucial to understanding genotype behavior in response to different environments.

Keywords: oat protein isolate, globulin, SE-HPLC, LC-MS

4.2. Introduction

Consumption and interest in oat have been increasing due to the attractive health benefits it has to offer. Oat consumption has been associated with lowering blood cholesterol levels due to its high β-glucan content. Oat is also composed of a nutritionally balanced amino acid profile with a protein content of 15-20%, making it a good protein source (Mohamed et al., 2009). Currently, oat is often consumed as rolled oats in a variety of breakfast foods. Given the recent demand for protein ingredients, there is a need to find protein sources that are of high nutritional quality and are also sustainable. The relatively high protein content of oat among cereals makes it a strong candidate for use as a protein ingredient. Previous work discussed the classification of oat protein into four types of storage proteins including globulin, prolamin, albumin, and glutelin (Klose & Arendt, 2012). These proteins play a significant role in the functionality and characteristics of oat, and oat protein isolates and concentrates.

The determining factor for oat protein use in food applications is its functionality in different food systems. With regard to Osborne classification, oat protein consists of 70-80% globulins, 4-15% prolamins, 1-12% albumins, and <10% glutelins, which are soluble in saline solution, dilute alcohol solutions, water, and acids or bases, respectively (Ching-Yung Ma & Harwalkar, 1984; Peterson, 1978). Globulin proteins are made up of acidic and basic polypeptides linked by disulfide bonds giving oats superior solubility, foaming, and emulsification in acidic and alkaline conditions when compared to other grain proteins (Yue, Gu, et al., 2021b). All these aspects are important and make OPI an excellent and versatile protein source. However, these functional properties are dependent on both genotypic influences as well as the growing environment. Studies have been conducted to evaluate how G × E affects the grain quality characteristics of oat and found that groat percentage, grain weight, protein content, and β-glucan content were dependent on both

environment and genotype equally (Doehlert et al., 2001). However, from a protein perspective, the impact of G × E is yet to be examined.

The protein composition of cereals has become an important research area due to various reasons such as allergenicity, functional properties and contamination with other grains in field or during processing. Scientists have developed various analytical methods to characterize oat proteins since 1970 (Sunilkumar & Tareke, 2019). HPLC is a promising technique to analyze protein composition for the purpose of breeding programs, quality control and food safety, grain registration as well as for varietal identification (Bietz, 1983). Considering the relative composition, oat contains high globulin content with low prolamins (previously noted in Osborne classification), as opposed to wheat with prolamins as a major storage protein, which has been linked to wheat's celiac allergenicity (Hoffmanova et al., 2019). However, a recent study conducted using 162 oat cultivars from 20 countries reported that oat protein compositional analysis is a great method to identify the gluten contaminations of oat and found that higher number of the samples tested were contaminated with wheat, barley, or rye (Gell et al., 2021). Therefore, protein compositional analysis is an excellent tool to determine protein variations across cultivars and growing environments. However, from a functionality perspective, oat protein has not been studied to identify the relationship between the techno-functional properties and oat protein composition.

A previous study reported that oat proteins could be divided into three main categories when analyzed using HPLC based on the elution time, as polymeric globulin proteins, avenins, and soluble non-avenin proteins (Gell et al., 2021). Another study used SE-HPLC to assess how hydrolysis with differing hydrolysates would impact the molecular weight of the oat protein isolate (Nieto-Nieto et al., 2014b). However, as reported in a recent review covering 137 papers published

between 1970 and 2015, analytical methods for measurement of oat proteins, are concentrated on cultivar identification to improve the nutritional quality (Sunilkumar & Tareke, 2019). However, the authors stated the need for validation of oat protein characterizing methods using HPLC techniques. Mass spectrometry (MS) is commonly used to supplement SE-HPLC analysis, for speciation or detections of specific elements while being particularly useful for elements within high-protein matrices (Koplık et al., 2002). LC-MS is now commonly used to identify and characterize cereal storage proteins as it is a sensitive and versatile technique for protein identification (Dawson et al., 2018). The complexity of cereal seed proteomes demands a sensitive analytical approach. LC-MS is superior to chromatographic and electrophoretic techniques which have been used traditionally, because it combines separation and identification in a single step, provided that good genomic sequence databases exist for querying MS data.

The aim of this study is to determine the effect of G × E on the protein composition through SE-HPLC and LC-MS for further in-depth relative quantitation of different proteins in OPI. This study investigates three cultivars of oat grown in the Canadian Prairies. To the best of our knowledge, SE-HPLC and LC-MS have not been used to quantitatively analyze the effect of G×E on OPI protein composition.

4.3. Materials and methods

4.3.1. Materials

The experiment included grains from three most established cultivars (Summit, AC Morgan and CS Camden) which are widely grown in Canada. These samples were collected from Canadian prairies with the highest reported oat acreages (Brandon-Manitoba, Saskatoon- Saskatchewan and Lacombe -Alberta) in Canada from the 2020 growing year (Table 4.2. shows the changes in the environmental conditions where samples were collected).

4.3.2. Extraction of OPI

Oat samples were first dehulled using a CODEMA dehuller (Minneapolis, MN, USA) and milled using a centrifugal mill (Retsch mill, ZM 200, Retsch GmbH & Co, Germany). Oat flour was first defatted using hexane in a 1:5 (flour:solvent) ratio using a cold extraction method and defatted oat flour was then used to prepare OPI by the optimized AE-IEP method (Yung Ma, 1983). First, 1:5 (flour:solvent) oat flour was solubilized in distilled water and adjusted to pH 9.0 using 2 M NaOH. After stirring at room temperature for 1 h, the solution was centrifuged at 4000 g for 20 min at room temperature, the supernatant decanted, and the protein precipitated by adjusting to pH 5.3. Centrifugation was performed again at 4000 g for 20 min at room temperature, and the resulting protein pellet was mixed with water, adjusted to pH 7.0, and freeze dried to obtain OPI. The protein extraction was carried out in triplicates for each genotype-environment combination and the freeze-dried samples were kept at -20 °C until further analysis. The protein content of the freeze-dried OPI was measured through the micro Kjeldahl method (AACC method 46-13.01).

4.3.3. Relative Protein Composition Measured by Size Exclusion-High Performance Liquid Chromatography (SE-HPLC)

First, the running conditions and the mobile phase for OPI separation were optimized upon testing different mobile phases and column running conditions, which could provide the best spectrum. OPI was solubilized in HPLC grade water for 30 min to obtain a 1% (w/v) protein solution. Then the samples were centrifuged at 16200 g for 15 min and the supernatant passed through a 0.45 µl filter into an HPLC vial. The vials were placed in a Waters Acquity Arc^{TM} HPLC instrument and analyzed using optimized parameters (Gell et al., 2021). The mobile phase was prepared by mixing 50% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA) with 50% water, also containing 0.1% trifluoroacetic acid (TFA). All chemicals used were of HPLC grade. The SE

column (Agilent Advance Bio Sec 300A, 2.7 µm 4.6 × 300 mm) was washed for 60 min with water and then washed with the mobile phase for 30 min prior to the analysis. The column was used at room temperature, the injection volume was 10 µl at a flow rate of 0.350 µl/min. The proteins were detected at 210 nm using Waters 2998 PDA Detector. The Advance Bio SEC 300A Protein standards (bovine thyroglobulin (670 kDa), bovine γ-globulin (150 kDa), chicken ovalbumin (45 kDa), equine heart myoglobin (17 kDa), and angiotensin (1 kDa) were used to calibrate the column based on molecular weight. The percentage area under each fraction was used for statistical analysis.

4.3.4. Liquid Chromatography-Mass spectrometry (LC-MS) analysis of OPI

4.3.4.1. Protein digestion

Oat protein digestion was carried out using the In-Solution Tryptic Digestion Kit (Thermo fisher, 89895). The digestion had three steps, reduction and alkylation, dialysis and then digestion. Freeze dried protein samples were digested as per manufacturer instructions with minor changes including a dialysis step prior to digestion. Dialysis was carried out using dialysis cups with a 7 kDa MWCO regenerated cellulose membrane device (0.1 mL capacity Slide-A-Lyzer MINI dialysis device, ThermoFisher Scientific, Ottawa, ON, Canada) for 2 h against 100 mM ammonium bicarbonate (ABC), followed by a second dialysis step in the same buffer for 2 h, then overnight in fresh ABC at 4°C. The samples were then cleaned to remove any impurities using Pierce C18 spin columns (Pierce C18 reversed-phase resin, Thermo Scientific Pierce, Ottawa, ON, Canada) and then solvents were evaporated using Thermo Savant DNA 120 SpeedVac Concentrator (Thermo Scientific, Holbrook, NY).

4.3.4.2. Mass spectrometric analysis of digested samples

Digested OPI samples were resolubilized in 20 µl of mobile phase A, which was water containing 2% (v/v) ACN and 0.1 % (v/v) formic acid (FA). The samples were separated using a C18 column (18 cm fused silica column, 75 µm ID, packed with Luna C18, 5 µm beads, 100 Å pores) coupled directly to the mass spectrometer via a nano electrospray ionization source (ThermoFisher Scientific, San Jose CA). An ACN gradient of mobile phase A to 60% mobile phase B (0.1%, v/v, FA in ACN) was delivered at 300 nl/min over 63 min (Easy nLC1000, ThermoFisher Scientific, San Jose CA), with a total program length of 80 min. From 0 to 2 min, only mobile phase A was run at 300 nL/min flowrate. Then from 2- 30 min mobile phase B was added from 3% to 10% with the same flow rate. At 58, 63 and 64 min, mobile phase B was further increased to 20%, 60% and 100% at the same flow rate and run until 80 min with 100% B. Mass spectra were acquired in a hybrid quadrupole-Orbitrap mass spectrometer (Q-Exactive: ThermoFisher Scientific, Bremen, Germany). A survey scan acquired over the range m/z 400-1800 was followed by 12 MS2 scans of the most intense ions, with dynamic exclusion set to 20 s.

4.3.4.3. Data collection and analysis

Protein identification and simultaneous label-free quantification (LFQ) was performed using MaxQuant (v2.3.1), where search parameter settings were used as monoisotopic mass accuracy of ±20 ppm for the first search and ±4.5 ppm for the second; up to two missed cleavages for tryptic peptides; peptide charge up to +7; fixed modification of carbamidomethyl (Cys), and variable modification of oxidation (Met) and acetylation (N-terminus). Raw mass spectrometry data files were queried against the genomic sequences of *Avena sativa* (OT3098 v2, PepsiCo) downloaded from GrainGenes (https://wheat.pw.usda.gov/jb?data=/ggds/oat-ot3098v2-pepsico) in May 2023 (Kamal et al., 2022). The results generated from MaxQuant were analyzed using Perseus (v1.6),

which is a companion software to MaxQuant used for statistical analysis. The LFQ values generated by MaxQuant were loaded as main columns for statistical analyses. The matrix was then reduced by filtering out proteins only identified by site, potential contaminants (from the default list of common contaminants embedded in the software) and proteins with a reversed sequence (decoys). The intensity values were transformed logarithmically (log2) and rows were filtered based on valid values, with at least two required per row, i.e. each identified peptide had to have a valid LFQ value in at least two biological replicates to be included in the final data. In cases where LFQ values were missing, the missing value was imputed using random numbers generated from the Gaussian distribution of the existing values, but down-shifted by 1.8 standard deviations (width set to 0.5 SD) to mimic low abundance protein LFQ values more accurately (Emir et al., 2022).

4.3.5. Statistical analysis

Protein samples were extracted in triplicates and used for SE-HPLC and MS analysis. . Two-way analysis of variance with genotype and environment as fixed effects was conducted to determine the effect of genotype, environment, and G × E on oat protein composition analyzed through SE-HPLC and globulin composition analyzed through LC-MS. Treatment means were compared using Tukey's multiple comparison procedure at $\alpha = 0.05$ using the R software package (version 4.1.2). Principal component analysis (PCA) was performed using OriginPro (v2023) software to explore relationships among globulin protein levels and genotype-by-environment interactions.

4.4. Results and discussion

4.4.1. Relative Protein Composition

The SE-HPLC separation resulted in 7-8 distinguishable peaks as shown in Figure 4.1. Each chromatogram was divided into four fractions based on relative molecular weight such as

polymeric globulins, avenins, glutelins and albumins, and very small proteins as defined below. The peaks at elution time 4.5-9.0 min represent polymeric globulins and high molecular weight aggregates, up to 670 kDa in size. The second fraction is made of avenins eluting at 9.0-11.5 min, which contains proteins with molecular weight of 15-22 kDa. The peaks at elution time 11.5-13.3 min represent the small proteins and peptide mixture of glutelins and albumins, which falls at 1-15 kDa. The last fraction at 13.3-15.0 min is comprised of peptides, which are smaller than 1 kDa in size. The chromatograms had better separation with Agilent Advance Bio Sec 300A column compared to previously reported work, which might be due to differences in the protein extraction method as well as separation conditions (Gell et al., 2021). The area percentage indicates the relative abundance of each protein groups. Considering the area percentage under each peak, only environment had a significant effect whereas the peak area across genotypes remained similar to each other. These results suggest that the protein composition had a stronger effect from the environmental conditions rather than from the genotypic background.

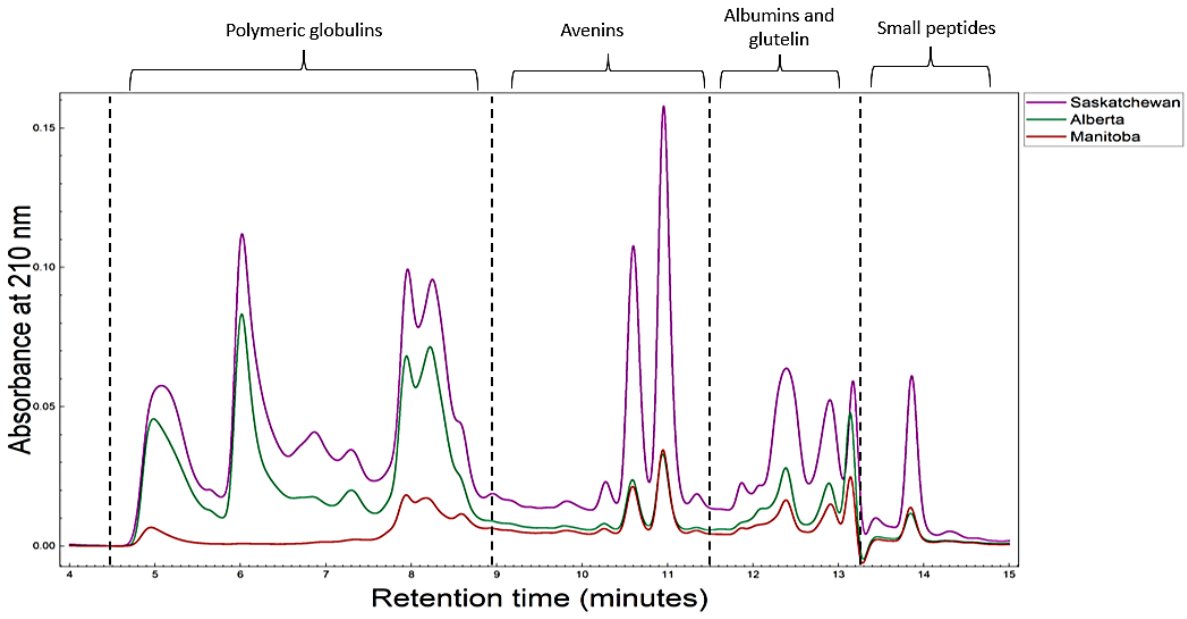
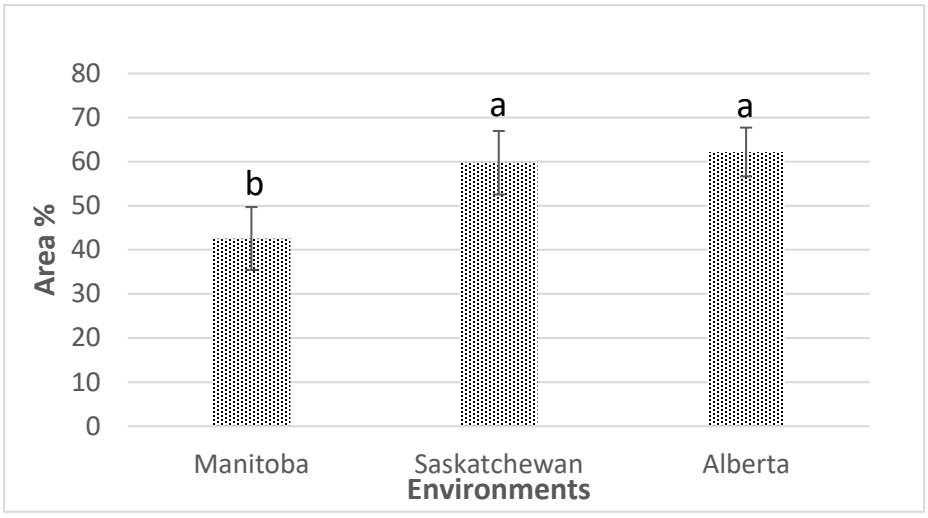


Figure 4.1. SE-HPLC chromatograms of the OPI sample Summit of Manitoba, Saskatchewan and Alberta at 210 nm UV absorbance

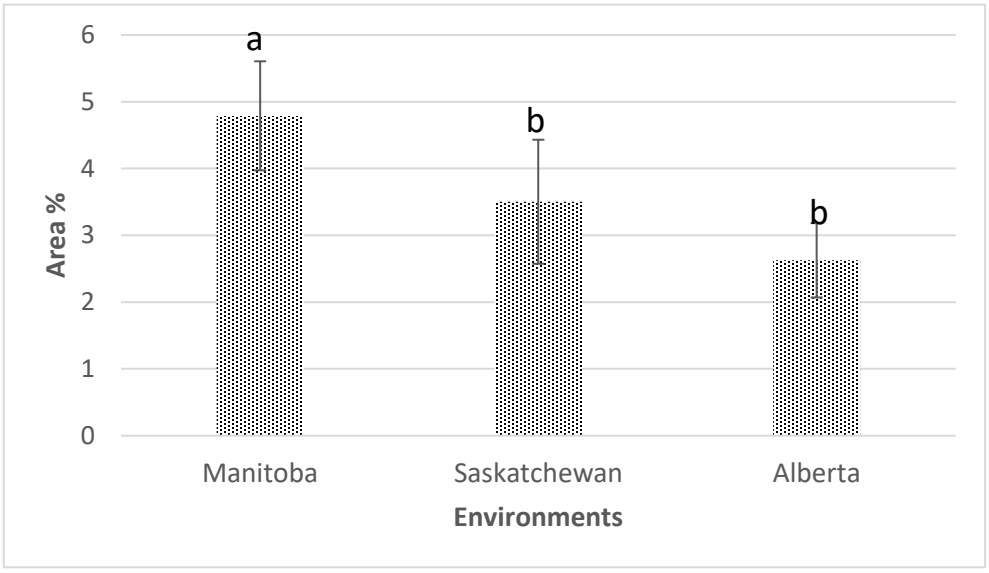
The first fraction is made up of globulins as well as the high molecular weight aggregates. According to previous studies, the majority of oat globulin exists as a hexamer, while a small amount can also exist as a mixture of acidic and basic polypeptides upon SE-HPLC separation (Y. Zhao et al., 2004). Considering the first fraction, samples from Alberta (62.2%) and Saskatchewan (59.8%) had higher area percentage for fraction one than that of Manitoba (42.6%). Previous studies reported that oat protein is comprised of a higher amount of globulin (50-80%) followed by prolamins, glutelins, and albumins, which is in agreement with the results of this study (Robert et al., 1985). Furthermore, in terms of relative area distribution in fraction one, samples from Manitoba had more peak area after retention time 7.5 min (at 45 kDa), which may indicate the presence of globulin as a mixture of acidic (36 kDa) and basic (22 kDa) polypeptides rather than as a dimer (58 kDa) (Chin-Yung Ma, 1985). However, samples from Alberta and Saskatchewan indicate a different chromatogram containing a large peak eluting at 6.0-6.5 min corresponding to the hexamer (330-350 kDa) of globulin (Nnanna & Gupta, 1996; Ching-Yung Ma & Khanzada, 1987). Considering all other fractions (2, 3, and 4), samples from Manitoba showed the highest area percentage, whereas samples from Saskatchewan and Alberta had no significant difference among them. In the light of results shown in Figure 4.2, a significant environmental effect has also been demonstrated in terms of avenin protein composition (fraction 2). The avenin protein fraction of all samples from Manitoba had an area of 28.7%, while Saskatchewan and Alberta had 19.3% and 16.9% respectively. The avenin protein fraction contains the alcohol soluble prolamin fraction of OPI, accounting for 4-14% of total protein (Shewry & Halford, 2002). An early study stated that the prolamin fraction of oat protein ranges between 15.5 – 23.5 kDa while a recent study reported the molecular weight of avenin as 20 to 36 kDa (Ching-Yung Ma & Harwalkar, 1984; Comino et al., 2016). In this study, the peaks between 15-22 kDa were selected as the avenin

protein fraction. In general, there were no visual differences in the avenin peaks among the tested samples as opposed to the changes observed for polymeric globulin fraction across the three environments.

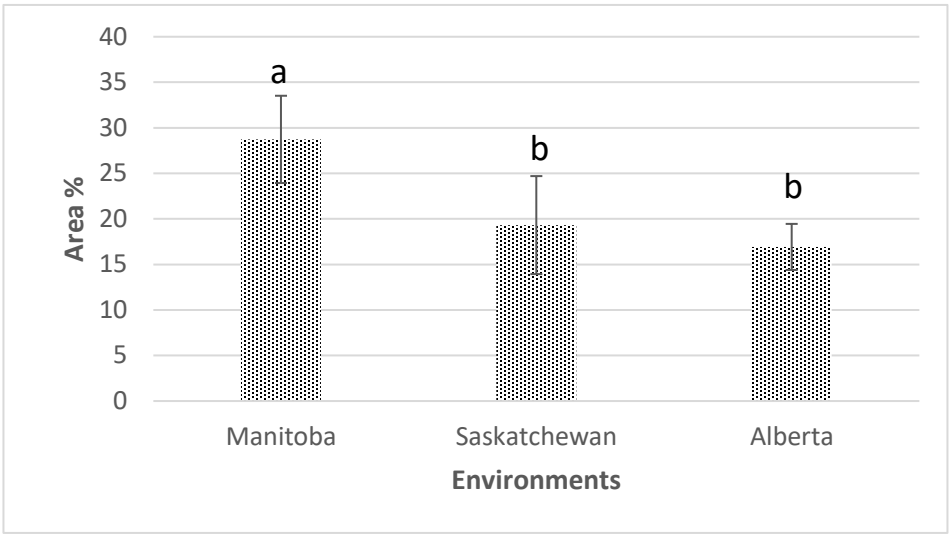
(a)



(b)



(c)



(d)

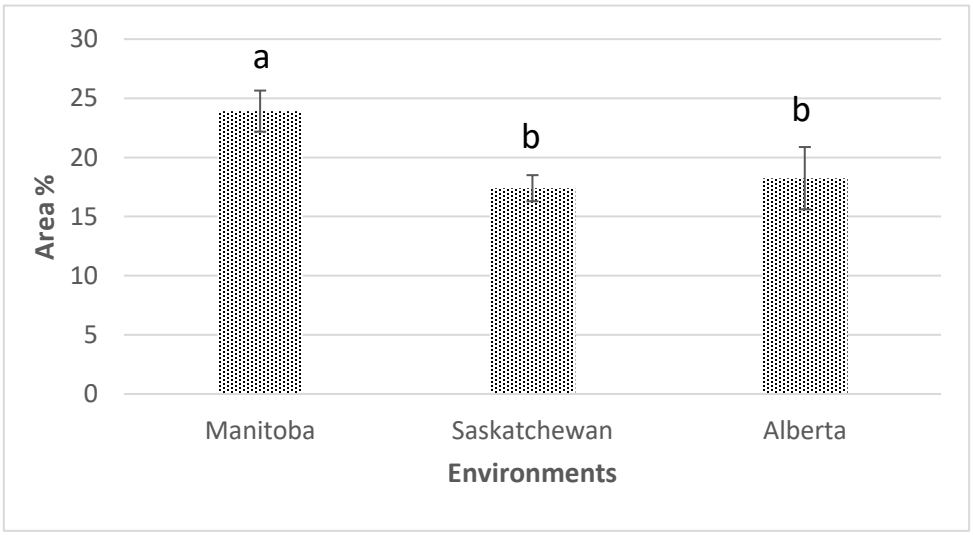


Figure 4.2. Changes in the percentage area differences across environments for the protein fractions. (The area percentage indicates the relative abundance of each protein fraction)

(a) Fraction 1 – Polymeric globulin
(b) Fraction 2 – Avenins
(c) Fraction 3 - Albumins and glutelin
(d) Fraction 4 – Small peptides

For the third protein fraction, glutelins and albumins, samples from Manitoba had an area of 23.9%, whereas Saskatchewan and Alberta had 17.4 % and 18.2%. According to previous studies, the proteins in the range of 6-18 kDa are mainly glutelins and albumins and in this study they are located in the 3rd fraction (Figure 1) between >1 kDa and <17 kDa (Chin-Yung Ma, 1985). Furthermore, considering the last fraction, which consist of peptides and likely of small contaminants (<1 kDa), samples from Manitoba had significantly higher area (4.8%) in comparison to that of Saskatchewan (3.5%) and Alberta (2.6%). In previous studies, the small peptides were not discussed due to their absence. Peaks corresponding to small peptides may appear if proteins undergo hydrolysis. A previous study reported that proteins in the range on 0.4-0.9 kDa appear in SE-HPLC analysis in OPI upon hydrolysis by trypsin, pepsin and alcalase (Nieto-Nieto et al., 2014b). In this study, the OPI were prepared through AE-IEP method, which might have caused some level of protein denaturation or dissociation given the use of alkaline solvents for protein extraction and hexane for oat flour defatting. This might be the reason for the presence of very small peptides and the possible contaminants that were co-extracted by HPLC. Previous studies reported that the reproducibility of chromatograms can be influenced by column temperature and other conditions and also by protein extraction conditions (Lapvetelainen et al., 1995). All OPI samples were extracted in triplicates for the analysis with SE-HPLC. Therefore, slight changes within the sample triplicates can be due to the differences that might occur during the extraction procedure.

Oat seed protein composition is unique among cereals due to a higher globulin to prolamin (avenin) ratio. Previous studies have reported that oat seeds contain about 10-11 times as much globulin as avenin (Boyer et al., 1992). Considering the globulin:avenin (G/A) ratio, only the environment had a significant effect on G/A while the effect of genotypes remained similar to each other (Figure

4.3). However, previous studies have not been conducted to evaluate the effect of G×E on G/A ratio. Previous studies have reported that legumin/vicilin ratio in pea protein is influenced by genotype and cultivation year (Mertens et al., 2012). In the present study, samples from Alberta and Saskatchewan had a higher G/A ratio compared to those from Manitoba. Usually in cereals, the prolamin fraction has a positive correlation with total protein content and increasing the prolamin fraction results in a poor amino acid composition and diminished nutritional quality (Mäkinen et al., 2016a). However, in rice and oats the globulin fraction increases with the total protein content, which in turn improve the nutritional quality of oat protein (Welch, 2011). Considering the protein content of OPI, samples from Manitoba and Alberta had higher protein content than samples from Saskatchewan. The OPI from AC Morgan grown in Manitoba had the highest protein content of 90.2% while OPI from Summit grown in Saskatchewan had the lowest protein amount of 76.9%. Therefore, a direct relationship between the globulin protein percentage and the total protein content could not be identified. However, from a nutritional standpoint, the globulin fraction is found to have a higher proportion of lysine, histidine and arginine compared to avenin fraction (Welch, 2011). Since lysine is considered to be the limiting amino acid in cereal grains, having high lysine content in oat protein could be useful for the application of plant based protein supplements for children and adults (Draper, 1973). Given its high higher G/A ratio, oat proteins extracted from samples in Alberta and Saskatchewan could be identified as superior in terms of nutritional quality than those of Manitoba. Furthermore, avenin proteins are found to trigger allergenicity in some individuals therefore, OPI with reduced avenin protein content has the potential to be used as a plant protein ingredient to replace celiac triggering wheat gluten and highly allergenic soy proteins in food systems (Mäkinen et al., 2016a).

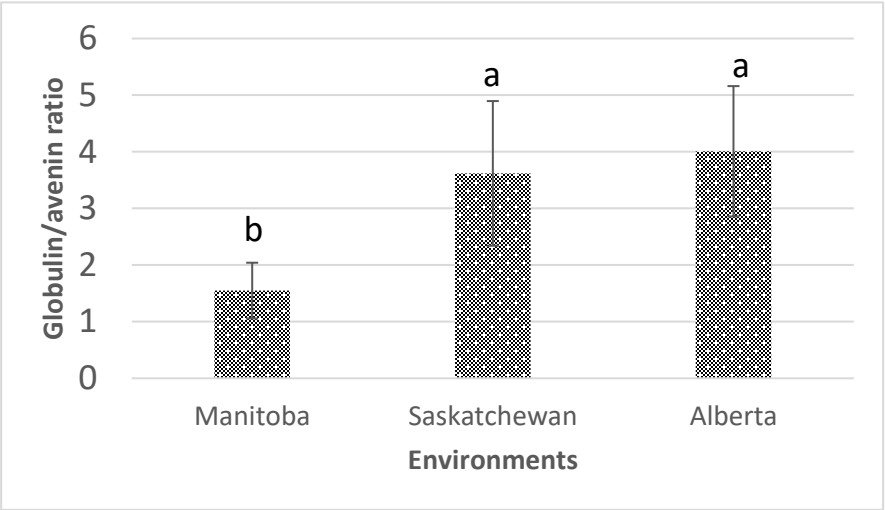


Figure 4.3. The changes in the globulin/avenin ratio across environments for the protein fractions

Soy glycinin can make strong and turbid gels upon thermal gelation and possess higher thermal stability (Damodaran & Kinsella, 1982). Oat globulin has a similar structure to soy glycinin, and this suggests that a higher G/A ratio could be beneficial in for novel product development using OPI (Ma & Harwalkar, 1987). Therefore, it can be suggested that oat proteins sourced from the tested cultivars in Alberta and Saskatchewan would be better candidates for food applications which require strong gelling properties of proteins such as for a meat analogue compared to oat proteins sourced from Manitoba regardless of the genotypes. Furthermore, the protein composition can be manipulated through carefully choosing the right growing environment for the production of oats with a specific G/A ratio for specific food industries. However, the selection of cultivars with a certain G/A is dependent upon their sensitivity towards environmental changes as demonstrated in the current study. Therefore, oat cultivars that are less sensitive to the effect of environmental conditions can be chosen for industries to provide proteins with a specific G/A ratio, hence good functionality.

4.4.2. Liquid Chromatography/Mass spectrometry (LC/MS) detection of peptides in OPI

In total 168 proteins were identified across all samples used in this study. The proteins identified were grouped into categories based on their abundance in OPI: globulins, prolamins/avenins, glutelins, enzymes/ albumins, enzyme inhibitors, heat shock proteins, grain softness proteins and allergenic proteins. Since the majority of OPI is composed of globulins (about 70 -80%), the globulin protein LFQ values were used for further analysis (Robert et al., 1983). In total 18 LFQ intensities associated with storage globulins were detected and three main types of globulin proteins were identified: P14812|SSG2-12S seed storage globulin, Q6UJY8_TRITU-globulin and M7ZQM3_TRIUA-Globulin-1 S allele. The LFQ intensities of these proteins across genotypes and environments (G and Es) are associated with their relative abundance as presented in Figure 4.4. The globulin protein with a mass of 62.0 kDa was observed with the highest LFQ protein intensity. Furthermore, a globulin protein with a mass of 56.8 kDa was observed with the second highest LFQ protein intensity. These results are in agreement with a previous study which reported that major oat globulins had molecular weight between ~53 – 62 kDa (Walters et al., 2018). The structure of wheat globulin-1 S allele has been characterized in a recent study which reported that globulin-1 S allele is one of the most abundant glycoproteins in wheat and has a potential allergenicity due to its IgE-binding capacity (Zhu et al., 2022). Future studies are needed to assess the quantity of globulin-1 S allele in OPI, which could be a potential allergen.

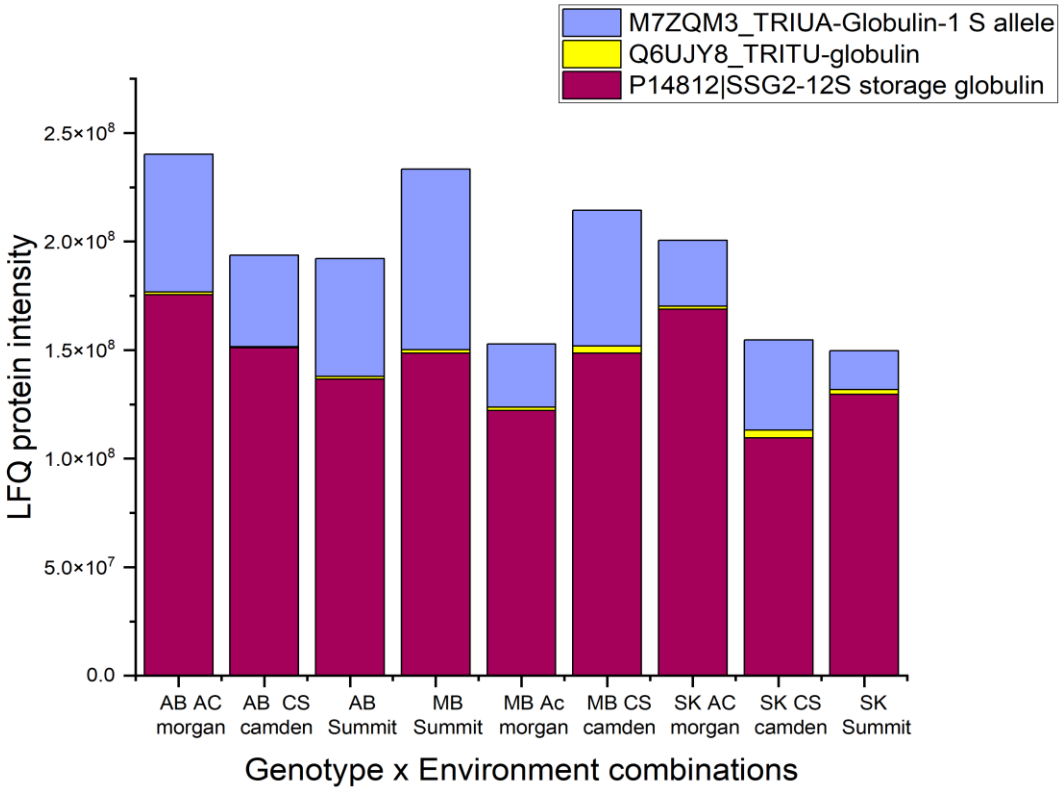


Figure 4.4. Variations in globulin protein composition across genotypes and environments as per LC-MS/MS analysis

A total of 5 avenin proteins were detected during the analysis which had the molecular weight in the range of ~17-29 kDa with the most abundant avenins having a MW of 28.9 kDa and 27.8 kDa (Table 4.1.). This is in agreement with previous work which reported 25.9 kDa for oat avenins (Walters et al., 2018). The glutelin fraction was also detected as a total of 3 proteins even though it is considered to be present in very low quantities in OPI. Previous work has described that glutelins serve a metabolic and/or structural role in oat and are therefore present only at low abundance, accounting for <10% of total proteins (Robert et al., 1983). In the present study, the most abundant glutelin was found to have 58.5 kDa molecular weight.

Table 4.1. Composition of the major proteins in the OPI by LC-MS/MS analysis.

Protein name	Protein ID	Molecular weight (kDa)	Sequence coverage %
12S seed storage globulin	P14812|SSG2_AVESA	62.037	83.8
Avenin protein	F2Q9W5_AVESA	28.947	27.6
Avenin protein	I4EP88_AVESA	27.839	36.8
Glutelin	Q0E261_ORYSJ	58.513	74.1
Saposin-like aspartyl protease family protein	AT1G62290.2	33.578	59.3
Chitinase family protein	AT2G43610.1	25.823	27.3
Gamma interferon inducible lysosomal thiol reductase family protein	Q10MT7_ORYSJ	28.141	33.7
Alpha-amylase/trypsin inhibitor	P01087|IAAT_ELECO	16.463	68.5
Avena alpha amylase trypsin inhibitor	A0A1B2LQE5_9POAL	16.925	67.5

Avena alpha amylase trypsin inhibitor	A0A1B2LQA8|A0A1B2LQA8_9POAL	15.915	60.8
17.6 kDa class II heat shock protein	AT5G12020.1	26.503	47.1
Heat shock 70 kDa protein	P11143|HSP70	71.596	48.5
Allergenic protein	Q8H4M4_ORYSJ	18.172	55.5
Grain softness protein	G8CLR3_HORMA	18.52	68.9
Grain softness protein	A8QRR4_TRITI	18.266	38

A total of 36 enzymes were detected during MS with saposin-like aspartyl protease family protein (33.6 kDa) and chitinase family protein (25.8 kDa) being the most abundant enzymes across all G and Es. However, in some G and Es, gamma interferon inducible lysosomal thiol reductase family protein (28.1 kDa) was also detected in highest LFQ values. These G and Es were, AB -CS Camden, MB- AC Morgan, SK-AC Morgan and SK-Summit. Considering the third most abundant enzyme across G and E, glyceraldehyde-3-phosphate dehydrogenase (36.6 kDa) was detected in all OPI samples. Among the enzymes the presence of Alcohol dehydrogenase, peroxidases, isomerase, glucosidases, branching enzymes and hydrolases were also detected.

Enzyme inhibitors can be found in all plants and regulate enzyme hydrolysis and also serve in the plant defense system (Mikola & Mikkonen, 1999). These inhibitors interfere with starch and protein digestion in insect guts by impeding digestive enzyme function (Breiteneder & Radauer,

2004). In this study 22 enzyme inhibitors were detected and were categorized into two main groups as trypsin and amylase inhibitors. The most abundant enzyme inhibitors found in all OPI samples were alpha amylase/trypsin inhibitors (ATIs) with a molecular weight within ~15.9-16.9 kDa range. A previous study on oat was able to detect a total of 25 ATIs and reported that they can act as allergens by contributing to intestinal inflammation (Tanner et al., 2019). Other enzyme inhibitors found in this study were serpins (42.7 - 43.2 kDa) and dextrinase inhibitors (16.7 kDa). Serpins are serine proteinase inhibitors and wheat serpins are known to be associated with immunoglobulin (Ig)E related allergies including bakers' asthma (Shewry, 2009). Another group of proteins detected during LC-MS/MS was heat shock proteins and a total of 8 were identified during the analysis. In the present study, the detected heat shock proteins can be divided into two major groups as high molecular weight (~70-80 kDa) and low molecular weight (16.9-27 kDa) proteins, with 4 detected in each category. Considering the LFQ intensity, the two most abundant proteins within G and E were 17.6 kDa class II heat shock protein (26.5 kDa) and HSP70 heat shock protein (71.6 kDa). In terms of allergenic proteins, one allergenic protein was detected: Q8H4M4_ORYSJ with a molecular weight of 18.2 kDa which found to be homologous to an allergenic protein from *Oryza sativa subsp. japonica* (Rice) when searched through Uniprot database (https://www.uniprot.org). This might be due to the possible contamination of oat with other grains in the field or during processing such as dehulling and milling of oat samples. Furthermore, two grain softness proteins were also found during the LC-MS/MS analysis: G8CLR3_HORMA (18.5 kDa) and A8QRR4_TRITI (18.3 kDa). Previous studies investigating the function of grain softness proteins in rice reported that these proteins are directly responsible for controlling grain hardness and may be useful in modifying the texture of grains (Krishnamurthy & Giroux, 2001). Moreover, the other proteins detected apart from major components as described

in table 4.1. are vromindoline, puroindoline, caleosin, stress-response protein, 40S/60S ribosomal proteins and, lipid-transfer proteins.

4.4.3. Analysis of the impact of G × E on Globulin

A 2-way ANOVA was used to understand the effect of genotype, environment and G×E on globulin proteins. The LFQ intensity was used as a measure of protein abundance and the most abundant globulin type detected in this study was P14812|SSG2-12S seed storage globulin which showed significant differences between genotypes. This means that the expression of P14812|SSG2-12S seed storage globulins is significantly more impacted by genotype rather than the growing environment. These results could be linked to our previous work on oat protein structural characterization through SDS-PAGE which also revealed that the electrophoretic profile of oat proteins was strongly influenced by genotype rather than the environment (Mel et al., 2023). Samples from AC Morgan and CS Camden had a higher amount of P14812|SSG2-12S seed storage globulin than Summit as shown in Figure 4.5. According to previous work on proteins in oat bran, it has been reported that P14812 protein ID belongs to the 12S seed storage globulin group which also contains P12615, O49258, O49257 and Q38781 which however were not detected in the current study (Vanvi & Tsopmo, 2016). The Q6UJY8_TRITU-globulin had no significant effect from either genotype or environment. According to Figure 4.3, Q6UJY8_TRITU-globulin is present in smaller quantities compared to 12S seed storage globulin and Globulin-1 S allele protein. However, previous work have not reported Q6UJY8_TRITU-globulin under oat globulins.When searched through Uniprot, it is suggested to be from Poulard wheat (*Triticum turgidum)* while previous work on common wheat (*Triticum aestivum)* has listed Q6UJY8_TRITU-globulin as a protein identified through proteomics analysis of common wheat (Parit et al., 2018). Therefore, the occurrence of Q6UJY8_TRITU-globulin in minor quantities

might be due to possible contamination with wheat as the processing was not done in a wheat-free setting. The M7ZQM3_TRIUA-Globulin-1 S allele was significantly impacted by the environment and G × E. In terms of the environment, samples from Manitoba and Alberta had significantly higher amount of M7ZQM3_TRIUA-Globulin-1 S allele than Saskatchewan. Furthermore, in terms of G × E, only the genotypes within Manitoba showed significant differences among each other; Summit and CS Camden had significantly higher M7ZQM3_TRIUA-Globulin-1 S allele content compared to that of AC Morgan (Figure 4.6).

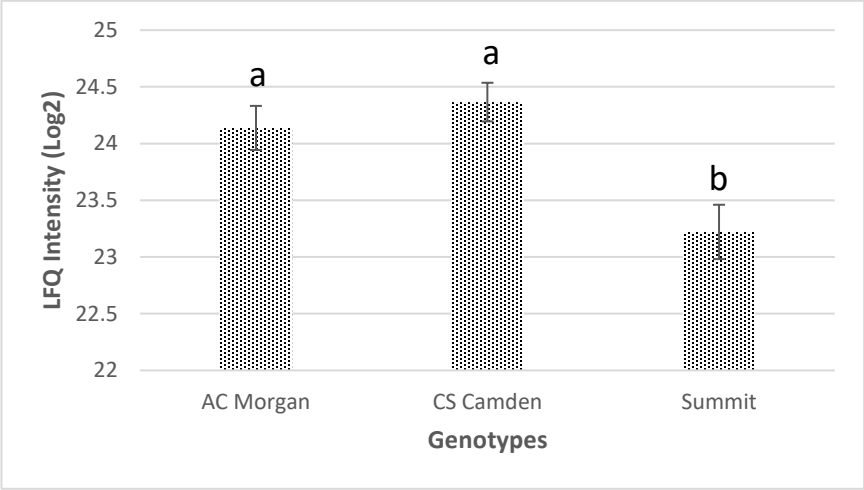


Figure 4.5. LFQ intensity of P14812|SSG2-12S seed storage globulins measured in three genotypes (AC Morgan, CS Camden and Summit). Mean value with different letters indicates a significant difference at the $P < .05$ level

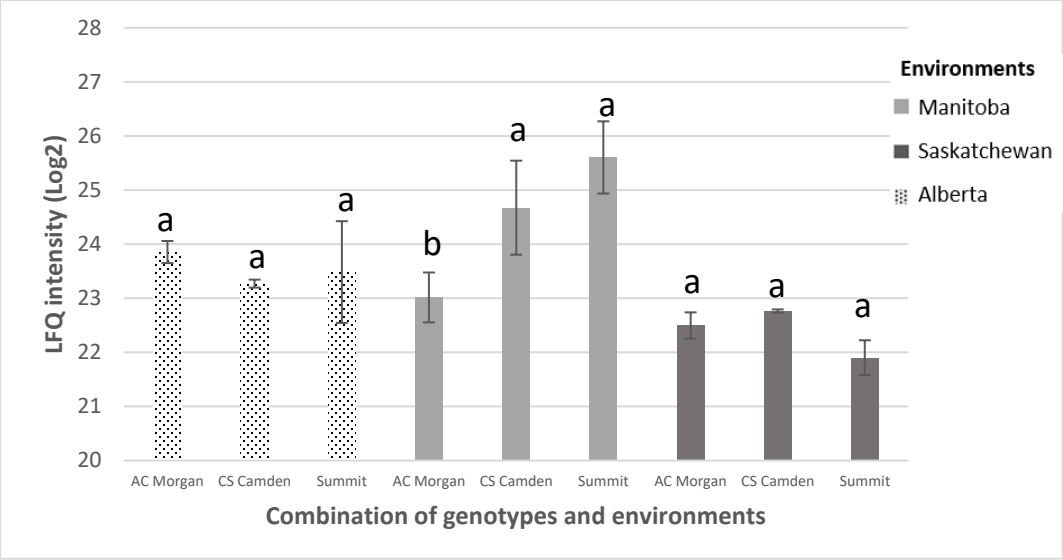


Figure 4.6. LFQ intensity of M7ZQM3_TRIUA-Globulin-1 S allele measured on three genotypes (Summit, AC Morgan, and CS Camden) in each of three environments (Manitoba, Saskatchewan, and Alberta). Mean value with different letters indicates a significant difference at the $P < .05$ level within an environment.

4.4.4. Principal component analysis of G × E on globulin composition

The PCA plot in Figure 4.7 illustrates the effect of G × E on the globulin proteins present in OPI. The percentage of the total variance explained by PC1 and PC2 is 41.7 % and 31.4 %, respectively, accounting for 73.1% of the total variance. In total, three different globulin types are represented by PC1 and PC2 including P14812|SSG2-12S seed storage globulin, Q6UJY8_TRITU-globulin and M7ZQM3_TRIUA-Globulin-1 S allele. The PCA scores clearly indicate that most of the protein replicates corresponding to the field reps cluster together. The PCA scores show that P14812|SSG2-12S seed storage globulin and M7ZQM3_TRIUA-Globulin-1 S allele are positively loaded to PC1 while Q6UJY8_TRITU-globulin is negatively loaded to PC1, reflecting the proteins that are positively and negatively correlated with PC1. The association of P14812|SSG2-12S seed

storage globulin with PC2 is negligible while the other two globulins are positively loaded to PC2, reflecting a positive correlation with PC2.

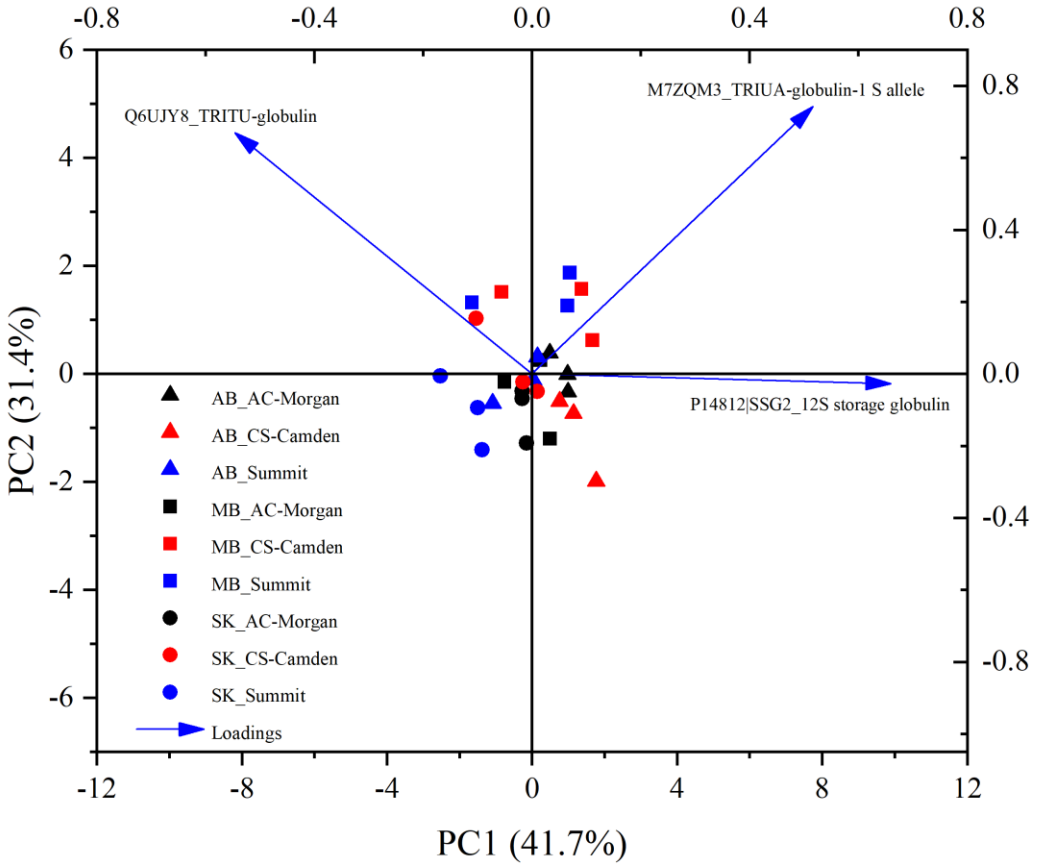


Figure 4.7. Principal component analysis score plots for principal component 1 and 2 for the LFQ protein intensities of globulins detected through LC-MS/MS. Three replicates were plotted for each G×E combination and each environment is represented by a common symbol, (□ – Manitoba, ○-Saskatchewan and, Δ- Alberta) and each genotype is represented by a common color (black -AC Morgan, blue – Summit and, red – CS Camden).

Considering the environments, samples from Manitoba seems to have a positive correlation with PC2 while samples from Alberta and Saskatchewan have a negative association with PC2. Therefore, samples from Manitoba have a positive association with M7ZQM3_TRIUA-Globulin-1 S allele and Q6UJY8_TRITU-globulin while samples from Alberta and Saskatchewan have a

negative association with them. These results can be linked with the 2-way ANOVA results (Figure 4.6.), which also indicates that samples from Manitoba having a higher content of M7ZQM3_TRIUA-Globulin-1 S allele than those from Saskatchewan, which, however, are not significantly different from those from Alberta. Considering the HPLC results (Figure 4.2a.) The polymeric globulin fraction was significantly impacted by the growing environment, and furthermore, samples from Alberta and Saskatchewan had significantly higher globulin concentration compared to that of Manitoba. Furthermore, considering 11S globulin of soy, it is reported that changes in environment had a greater effect on expression of 11S globulin compared to the variation observed among the soybean genotypes at any particular environment (Natarajan et al., 2016). According to previous studies, the similarity between the functionality of oat globulins and soy glycinin is mainly due to the structural similarity of oat 12S globulin to soy glycinin (Ercili-Cura et al., 2015). Therefore, a positive association of certain environmental conditions with P14812|SSG2-12S seed storage globulin needs further exploration in order to ascertain the best growing conditions for targeted markets, such as oat proteins. In this case, the samples from Alberta which cluster in the same quadrant as P14812|SSG2-12S seed storage globulin needs to be explored further.

In relation to genotypes, CS Camden has a positive association with PC1 specifically Alberta- CS Camden which has all three replicates clustered at the positive side of PC1 meaning that they have a positive association with P14812|SSG2-12S seed storage globulin and M7ZQM3_TRIUA-Globulin-1 S allele. Furthermore, when considering Figure 5. similar results can be observed which exhibit CS Camden having higher content of P14812|SSG2-12S seed storage globulin which is similar to AC Morgan but significantly higher than Summit. The PCA clearly shows that sample Saskatchewan- Summit has a negative association with PC1 and these results are also in

agreement with the 2-way ANOVA results which shows that Summit OPI contains significantly lower P14812|SSG2-12S seed storage globulin and Saskatchewan-Summit has relatively lower M7ZQM3_TRIUA-Globulin-1 S allele (Figures 4.5 and 4.6, respectively). AC Morgan shows somewhat negative association with PC2 which indicates a negative association with M7ZQM3_TRIUA-Globulin-1 S allele and Q6UJY8_TRITU-globulin. Furthermore, sample Saskatchewan-AC Morgan exhibits that all three protein replicates cluster with a negative correlation to M7ZQM3_TRIUA-Globulin-1 S allele. Certain environment-genotype combinations may increase the likelihood of expressing allergenic protein, M7ZQM3_TRIUA-Globulin-1 S allele such as Manitoba- CS Camden, Manitoba- Summit and Alberta – AC Morgan. Furthermore, a study conducted to investigate the celiac triggering ability of non-gluten proteins in wheat reported that a protein similar to globulin-1 S allele was detected in the tested wheat cultivars, hence globulins in wheat can also contain homologs of celiac disease epitopes (Lakhneko et al., 2020). According to a previous study, expression of 12S globulin had a significant effect from heat stress during the grain filling period than 2S globulins in rapeseeds, which showed decreases in the 12S globulin concentration under heat stress (Brunei-Muguet et al., 2015). However environmental stress conditions were not recorded in the 2020 growing year from which the samples for this study were collected (Table 4.2) and, in fact the western Canadian oat production was at its highest in 2020 since 2009 (Prarie Oat Growers Association, 2022). Therefore, the changes in oat globulin composition of a certain genotype across the environments tested could be a result of factors including soil, fertilizer input, precipitation or light radiation.

Table 4.2. The changes in the environmental conditions from May to August where samples were collected (Adopted from : Government of Canada, 2021)

Environment	Month	Elevation (m)	Mean temperature (°C)	Mean precipitation (mm)
Lacombe- Alberta	May	860.00	9.6	2.58
	June		13.9	3.04
	July		15.8	3.20
	August		15.8	1.65
Brandon- Manitoba	May	409.30	10.2	0.37
	June		17.4	7.38
	July		20.0	1.90
	August		18.3	1.88
Saskatoon- Saskatchewan	May	504.10	11.1	1.36
	June		15.3	3.56
	July		18.9	1.68
	August		18.0	0.52

Furthermore, previous studies have found that nitrogen fertilizer application could also influence the proportion of albumin, globulin, glutenin and gliadin in wheat (Shi & Liu, 2013). However, similar information is not available for oat globulins and, future studies are needed in this area to evaluate how fertilizer application, precipitation and light radiation affects the globulin composition in oat. In the current study, we observed that genotype AC Morgan and CS Camden could be better than Summit and in terms of environments, Alberta and Saskatchewan could be

better choices than Manitoba for OPI with more P14812|SSG2-12S seed storage globulin and less M7ZQM3_TRIUA-Globulin-1 S considering their association with G and Es. Furthermore, the G and Es having positive associations with P14812|SSG2-12S seed storage globulin could be used to develop new cultivars with better protein functionalities for targeted applications.

4.5. Conclusion

The protein quality of OPI is governed by the genotype as well as the environment. Oat growers are inclined towards specific oat cultivars due to high yield, disease resistance, β-glucan content and agronomic features such as drought tolerance. However, these selected cultivars might not be the ideal selections in terms of their protein quality from a functionality perspective. The current study investigated the protein composition through SE-HPLC and LC-MS to understand the effect of G × E on oat protein composition as a whole and the globulin protein composition using three widely grown oat genotypes across the Canadian Prairies. The study showed that relative oat protein composition was mainly influenced by the growing environment, which indicated that Alberta and Saskatchewan as having higher polymeric globulin content than Manitoba and vice versa for the avenin fraction for the samples analyzed in this study. Furthermore, three main types of globulin proteins were identified through LC-MS including P14812|SSG2-12S seed storage globulin, Q6UJY8_TRITU-globulin and M7ZQM3_TRIUA-Globulin-1 S allele where P14812|SSG2-12S seed storage globulin was the most abundant of the three types across all genotypes and environments. Genotype had a significant effect on P14812|SSG2-12S seed storage globulin and AC Morgan and CS Camden had significantly higher quantity of 12S seed storage globulin in comparison to Summit. M7ZQM3_TRIUA-Globulin-1 S allele was significantly impacted by G×E and sample Manitoba-Summit and Manitoba-CS Camden showed somewhat higher M7ZQM3_TRIUA-Globulin-1 S allele concentration than the other samples. The PCA

analysis revealed that AC Morgan and CS Camden are positively associated with P14812|SSG2-12S seed storage globulin compared to Summit and in terms of environments, the samples grown in Alberta and Saskatchewan had a positive association with P14812|SSG2-12S seed storage globulin than Manitoba meaning these G and Es could be a better choice for OPI with more P14812|SSG2-12S seed storage globulin and less M7ZQM3_TRIUA-Globulin-1 S. The study highlights that different proteins have varying levels of positive and negative associations with genotypes and environments, highlighting the need for more elaborate studies to fully understand how G×E impacts oat protein structure-function relationships, especially as OPI is becoming a widely used ingredient in the food industry. This information is critical for the development of oat cultivars for targeted applications, such as those in the protein ingredient realm.

4.6. References

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Chapter 5. Conclusions and future research directions

Oat protein structural and functional characteristics are influenced by both genotypic and environmental conditions. The thesis is composed of two studies where in the first study, the impact of G × E on oat protein structural and functional properties were evaluated, while the second study was conducted to evaluate the relative protein composition through HPLC and LC-MS techniques.

Summary of the findings of the 1st study- Impact of G × E on Oat Protein Structural and Functional Characteristics

- Oat protein content is significantly impacted by genotype, environment and G × E
- The highest protein content was observed in OPI from AC Morgan grown in Manitoba with 90.2% and the lowest was from Summit grown in Saskatchewan with 76.9%
- SDS-PAGE results indicated the electrophoretic profile of oat proteins was strongly dependent on the genotype rather than the environment
- The environment had a significant effect on the protein surface hydrophobicity with samples from Alberta showing the highest surface hydrophobicity
- The denaturation temperature of all OPI samples ranged from 110.2 °C to 111.6 °C
- Denaturation enthalpy had a significant effect from genotype, environment and G × E where the average enthalpy of OPI ranged from 0.69 to 1.53 J/g
- In FTIR analysis, genotype, environment, and G × E interaction had no significant effect on the area percentage of amide I and amide II bands
- Generally, all OPI samples had low aqueous solubility and ranged from 13-33 % based on Kjeldahl protein solubility analysis

- Foaming capacity had a significant effect from genotype, environment and G × E, where the highest foaming capacity was observed in samples from Alberta
- Foaming stability of OPI was impacted by genotype and environment, considering environmental effect, samples from Alberta and Saskatchewan had elevated foaming stability compared to Manitoba and genotypes followed the pattern; Summit ≥ AC Morgan > CS Camden
- Even though gelling capacity of OPI had a significant effect from genotype and G × E, all samples formed soft gels with minor visible differences upon heat treatment
- A significant effect from genotype and environment was observed in terms of emulsion capacity, where samples from Saskatchewan produced emulsions with the smallest droplet size (8.7 ±1.7 μm in diameter)
- All emulsions were very stable after 30 min and had no visible layer separation even after 2 hours even though growing environment and G × E significantly impacted emulsion stability.

Overall, this study showed that oat protein content, protein profile, and functional properties are significantly impacted by genotype, environment as well as G × E. The utilization of any plant protein ingredient is dependent on its functional properties. Therefore, as an example considering the results obtained for foaming capacity being highest in samples from Alberta -Summit, it can be concluded that oat proteins extracted from oat samples in Alberta could behave as better foaming agents regardless of the genotype. Therefore, in a broader context, these results suggest the potential to identify the best genotypes and environment combinations for oat protein ingredients for targeted applications. Furthermore, the structure-function relationship of OPI along

with G × E studies are essential to determine oat cultivars that can be used to develop oat protein ingredients with predictable functional properties when grown across multiple environments.

Summary of the findings of the 2nd study- Oat protein compositional analysis through HPLC and LC-MS techniques

- SE-HPLC separated OPI into 4 major fractions, namely, polymeric globulins, avenins, albumins and glutelins and, very small proteins which are smaller than 1kDa in size
- Considering the area percentage under each fraction, only environment had a significant effect on protein composition
- In the first spectral fraction, samples from Alberta (62.2%) and Saskatchewan (59.8%) had higher area percentage than that of Manitoba (42.6%)
- For other fractions (2, 3 and 4), samples from Manitoba showed the highest area percentage, whereas samples from Saskatchewan and Alberta had no significant difference among them
- Eight main proteins were identified based on their abundance in OPI through LC-MS: globulins, prolamins/avenins, glutelins, enzymes/ albumins, enzyme inhibitors, heat shock proteins, grain softness proteins and allergenic proteins
- Three main types of globulin proteins were identified during the study including P14812|SSG2-12S seed storage globulin , Q6UJY8_TRITU-globulin and M7ZQM3_TRIUA-Globulin-1 S allele
- A total of 5 avenin proteins, 36 enzymes, 22 enzyme inhibitors and 8 heat shock proteins were detected through LC-MS

- The most abundant globulin type in OPI was P14812|SSG2-12S seed storage globulin and its expression was significantly impacted by genotype rather than the growing environment while it appears that the expression of M7ZQM3_TRIUA-Globulin-1 S allele is significantly impacted by environment as well as G×E

The PCA results indicated a positive association of AC Morgan and CS Camden with P14812|SSG2-12S seed storage globulin compared to Summit and in terms of environments, Alberta and Saskatchewan showed a positive association with P14812|SSG2-12S seed storage globulin in comparison to Manitoba. Overall, the results of SE-HPLC revealed that the relative OPI protein composition was mainly dependent on the environment rather than the genotypes, however the LC-MS results on globulins revealed that the most abundant globulin type in OPI (P14812|SSG2-12S seed storage globulins) was significantly impacted by genotype. Furthermore, considering the outcomes of the previous chapter, SDS-PAGE results indicated electrophoretic profile of oat proteins was strongly dependent on the genotype rather than the environment which is in alignment with the fundings from the LC-MS analysis. Furthermore, PCA results also demonstrated that certain environment and genotypic combinations could perform better in terms of yielding specific types of globulin proteins.

Future research directions:

This study was based on OPI extracted from oat samples obtained from the 2020 growing season, however the structural and functional properties could also vary across different years (i.e., environmental conditions). Therefore, assessment of samples across many years is important to identify changes in structural and functional properties of oat protein due to changes in environmental conditions such as precipitation, sun light and drought conditions. This is of specific importance given climate change induced changes in growing seasons. Therefore, further research

is needed to evaluate how protein structural and functional properties are affected by the environmental factors over many growing years. Furthermore, this study was conducted using field sample replicates taken from each location of Brandon-Manitoba, Saskatoon-Saskatchewan, and Lacombe-Alberta. Future studies are needed to evaluate the impact of G × E if the samples are taken from several locations from each province in the prairies as environmental conditions can vary widely within each province. Another extensive investigation can be done focusing on the correlation between structural and functional properties of OPI, where samples from many different oat cultivars from multiple locations can be examined for structural and functional properties. As an example, the results of this study indicated that samples from Alberta having higher protein surface hydrophobicity can be the reason for OPI from Alberta having elevated foaming capacity. As such, information about the correlation between structural properties and functionality are important aspects to consider when developing protein ingredients for targeted applications. Furthermore, oat protein chemistry can also be studied further to identify specific peptide sequences important for specific functional properties. Detailed amino acid analysis coupled with mass spectrometry could be useful to characterize amino acid composition and well as to identify specific amino acid focused characteristics, such as hydrophobic/ hydrophilic amino acid ratio and amino acid compositional changes across the genotypes and environments.

The nutritional quality of the OPI was not studied in this work. The nutritional quality of a protein ingredient is an important factor in addition to its techno-functionality when developing a protein ingredient to be used as a major protein source in any food system such as in meat analogs or infant foods. Therefore, *in-vitro* or *in-vivo* digestibility as well as bioavailability studies are needed to evaluate how G×E impacts oat protein digestibility and protein nutritional quality. Even though oat protein is regarded as a hypoallergenic protein compared to soy protein and wheat gluten, oat

protein is found to trigger celiac related symptoms in some individuals. As per the results from HPLC analysis, OPI contains a significant amount of oat avenins which can cause allergenicity. Therefore, from a food safety standpoint the allergenicity of OPI should also be tested specifically for celiac triggering epitopes. Furthermore, apart from avenins other possible allergenic proteins were also detected in the LC-MS study (e.g. M7ZQM3_TRIUA-Globulin-1 S allele) and future studies are needed to evaluate the allergenicity of specific oat proteins and also specific cultivars. Given its superior foaming properties of OPI from Alberta, new plant-based food applicates could be designed including replacers for egg white in meringue and baked goods. As per the results in this study oat protein can act as a good foaming agent and emulsifier, therefore, further studies are needed to evaluate the applicability and consumer acceptance of OPI in food systems including bakery items, salad dressings and chocolate etc. However, in terms of gelling properties of OPI, enzymatic modification can be used to develop OPI as a modified gelling and thickening ingredient to be used in food systems. Enzymatic modification can also be used to enhance the aqueous solubility of OPI. Furthermore, studies can also be conducted for nutraceutical development using OPI as a carrier or vehicle for sensitive bioactive compounds. Given its high denaturation temperature, oat protein can be used in products that undergo heat treatment yet need the functionality to be intact after processing. Therefore, OPI can specifically be used in nutraceutical applications which involve heat treatments such as spray drying or vacuum drying.

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