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Development of High-Protein Oat for the Feed and Food Industry

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PO Box 117
221 00 Lund
+46 46-222 00 00



Development of High-Protein Oat for the Feed and Food Industry

BINDU A. SUNILKUMAR

FOOD FOR HEALTH SCIENCE CENTRE | LUND UNIVERSITY



Development of High-protein Oat for the Feed and Food Industry

Bindu A. Sunilkumar



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Doctoral dissertation

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Faculty opponent

Professor Ulf Svanberg

Biology and Biological Engineering, Food and Nutrition Science
Chalmers University, Gothenburg, Sweden

Organization LUND UNIVERSITY Applied Nutrition and Food Chemistry and Food for Health Science Centre Author(s): Bindu A Sunilkumar	Document name	
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<p>Oats are an excellent source of high-quality proteins, with a favourable amino acid composition. Oat proteins therefore have great potential to complement existing animal and plant protein sources, especially if the protein content could be increased. In this thesis, high-protein oat lines were identified and characterised. More than a thousand individual lines in a mutagenised oat population (Targeting Induced Local Lesions in Genomes (TILLING) population) were screened for total protein using an elemental particle analyser. This identified 230 lines with a seed protein content of 15% protein or higher. Belinda, the original variety from which the mutagenised population was constructed, had approximately 12%, and the most protein-rich line had 24%. The amino acid compositions for 31 of the high-protein lines were determined by various methods, and the contents of essential amino acids (EAA) were evaluated according to the FAO/WHO amino acid recommendations. This showed that several of the high-protein lines contained sufficient levels of EAAs, although there was some variability in the amounts of nutritionally limiting amino acids. Several lines had higher EAA levels than Belinda. Five of the high-protein oat lines were selected for asymmetric flow field-flow fractionation (AF4) analysis. In all experiments the AF4 instrument was connected to an online multi-angle light scattering (MALS) and ultraviolet (UV) detection system. Greater variation was found in the quantity of soluble proteins in the different high-protein lines than in Belinda, and a few lines had clearly elevated levels of globular proteins. Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) also revealed that the increase in protein in the high-protein lines was mainly due to an increase in the globulin fraction. The effect of heat treatment on the soluble protein content in oat groats was also evaluated, using the AF4 system and amino acid analysis. The results showed that the total amount of soluble protein was reduced by 50%, mainly due to a reduction in amino acids associated with albumin and water soluble prolamins. An attempt was made to increase protein content by nitrogen fertilisation, and effects on grain quality were also analysed using avenin protein of Belinda oat cultivars grown or developed under various fields and in the greenhouse as a reference. Results showed that excess nitrogen fertilisation (≥ 100 kg/ha) increased total protein percentage in Belinda and high-protein oat lines. Furthermore, oat lines grown under field conditions showed differences in avenin proteins when compared to the corresponding lines grown in the greenhouse, as analysed by SDS-PAGE gel electrophoreses. This may explain the differences in protein levels in cultivars grown under different environmental conditions. Crosses were performed between the six lines with the highest protein levels and the original non-mutated Belinda variety from which the mutagenised population was derived. The F1 hybrid seeds were grown in a greenhouse, and self-pollinated and individual seeds from the F2 offspring were analysed. This showed that the high-protein character was stably inherited. To test this further, the 15 high protein lines were amplified in several different plots in the field and the protein content was again determined in seeds harvested at the end of the season. This confirmed the stability of the high-protein character. Total dietary fibre (TDF), β-glucan and lipid levels were also measured in the selected lines. The analysis showed that the values for these components were normally distributed around the original level in Belinda, i.e. there was no positive or negative correlation between fibre, β-glucan, lipid and high protein content. In conclusion, the high-protein oat lines, identified here from an oat mutagenised population, proved to be phenotypically stable in the field and produced high-quality proteins. When developed further, the resulting cultivars will be very valuable for future use in the food and feed industry. Total dietary fibre, especially soluble and insoluble fractions and βglucans, is high in these lines, which is another important benefit in the use of high-protein oat lines for food applications. Since oats in general give good yields and quality, especially in the Nordic countries, the lines presented here have potential to become a new source of vegetable proteins and will enable the development of novel food products based on oat.</p>		
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Development of High-protein Oat for the Feed and Food Industry

Bindu A. Sunilkumar



LUND
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Bindu A. Sunilkumar
Food for Health Science Centre
Faculty of Engineering, Lund University
Box 124, SE- 22100, Lund, Sweden, 2016

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amma,

my mind still talks to you....

my heart still looks for you.....

my soul knows you are at peace.....

Contents

Abstract	9
Popular science summary	11
List of Papers.....	14
My contribution to the papers	16
Abbreviations	17
Thesis at a glance	18
Chapter 1. Introduction.....	21
Oats	23
Oats as a functional food for health	24
Use of oat proteins as a feed ingredient	25
Nutrient composition and nutritional quality of oats in comparison with other cereals	26
Nutrient composition vs. nutritional quality	26
Proximate constituents.....	26
Protein quality	28
Dietary fibre components	29
Beta-glucan.....	30
Lipids.....	32
Micronutrient composition	33
Chapter 2. Oat storage proteins	35
Classification and solubility fractionation.....	35
Globulin.....	36
Avenin	36
Albumins and glutelins.....	37
Amino acid composition	37

Chapter 3. Application of oat proteins in industrial processes	39
Utility of oat protein functionality	40
Thermal stability	40
Gelling/coagulation	41
Flow properties	41
Protein concentrates.....	41
Chapter 4. The mutagenised oat population	43
Mutation breeding	44
Identification of novel traits in mutated populations.....	45
Chapter 5 Present work	47
Overall aim	47
Specific aims	47
Methodology	48
Protein quantification using Elemental Particle Analyser (EPA).....	48
Amino acid analyser.....	50
Asymmetric flow field-flow fractionation (AF4).....	50
Chapter 6. Results and discussion	53
Screening of oat TILLING population (Papers I-III).....	53
Amino acid analysis of high-protein oat lines (Paper II, III)	55
Soluble proteins of high protein oat lines (Paper III).....	57
Avenin proteins of mutated oat lines (Paper IV).....	58
Effect of heat treatment on soluble oat proteins (Paper V)	60
Concluding remarks.....	63
Future perspectives	65
Acknowledgements	67
References	71

Abstract

Oats are an excellent source of high-quality proteins, with a favourable amino acid composition. Oat proteins therefore have great potential to complement existing animal and plant protein sources, especially if the protein content could be increased. In this thesis, high-protein oat lines were identified and characterised. More than a thousand individual lines in a mutagenised oat population (Targeting Induced Local Lesions in Genomes (TILLING) population) were screened for total protein using an elemental particle analyser. This identified 230 lines with a seed protein content of 15% protein or higher. Belinda, the original variety from which the mutagenised population was constructed, had approximately 12%, and the most protein-rich line had 24%.

The amino acid composition for 31 of the high-protein lines was determined by various methods, and the content of essential amino acids (EAAs according to FAO/WHO) was evaluated according to the FAO/WHO amino acid recommendations. This showed that several of the high-protein lines contained sufficient levels of EAAs, although there was some variability in the amounts of nutritionally limiting amino acids. Several lines had higher EAA levels than Belinda.

Five of the high-protein oat lines were selected for asymmetric flow field-flow fractionation (AF4) analysis. In all experiments the AF4 instrument was connected to an online multi-angle light scattering (MALS) and ultraviolet (UV) detection system. Greater variation was found in the quantity of soluble proteins in the different high-protein lines than in Belinda, and a few lines had clearly elevated levels of globular proteins. Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) also revealed that the increase in protein in the high-protein lines was mainly due to an increase in the globulin fraction.

The effect of heat treatment on the soluble protein content in oat groats was also evaluated, using the AF4 system and amino acid analysis. The results showed that the total amount of soluble protein was reduced by 50%, mainly due to a reduction in amino acids associated with albumin and water soluble prolamins.

An attempt was made to increase protein content by nitrogen fertilisation, and effects on grain quality were also analysed using avenin protein of Belinda oat cultivars grown or developed under various fields and in the greenhouse as a reference. Results showed that excess nitrogen fertilisation (≥ 100 kg/ha) increased total protein percentage in Belinda and high-protein oat lines. Furthermore, oat lines grown under field conditions showed differences in avenin proteins when compared to the corresponding lines grown in the greenhouse, as analysed by SDS-PAGE gel

electrophoreses. This may explain the differences in protein levels in cultivars grown under different environmental conditions.

Crosses were performed between the six lines with the highest protein levels and the original non-mutated Belinda variety from which the mutagenised population was derived. The F1 hybrid seeds were grown in a greenhouse, and self-pollinated and individual seeds from the F2 offspring were analysed. This showed that the high-protein character was stably inherited. To test this further, the 15 high protein lines were amplified in several different plots in the field and the protein content was again determined in seeds harvested at the end of the season. This confirmed the stability of the high-protein character.

Total dietary fibre (TDF), β -glucan and lipid levels were also measured in the selected lines. The analysis showed that the values for these components were normally distributed around the original level in Belinda, i.e. there was no positive or negative correlation between fibre, β -glucan, lipid and high protein content.

In conclusion, the high-protein oat lines, identified here from an oat mutagenised population, proved to be phenotypically stable in the field and produced high-quality proteins. When developed further, the resulting cultivars will be very valuable for future use in the food and feed industry. Total dietary fibre, especially soluble and insoluble fractions and β -glucans, is high in these lines, which is another important benefit in the use of high-protein oat lines for food applications. Since oats in general give good yields and quality, especially in the Nordic countries, the lines presented here have potential to become a new source of vegetable proteins and will enable the development of novel food products based on oat.

Popular science summary

According to the Nordic Nutrition Recommendations (NNA) 2012, a protein intake corresponding to 10-20 percentage of total energy intake (E%) is recommended. The EFSA Panel, WHO and FDA have the same recommendation, and propose several positive health benefits as a result of an optimum protein intake. The range of 10-20 E% is necessary for infants (0-1 y), children (10-18 y) adults (18-64 y), elderly people (≥ 65 y) and pregnant and lactating women. This places great demands on the proteins in terms of, for example quality, source, and environmental sustainability. Overall consumption of meat and animal food products is constant, but there are many good reasons to use alternative, vegetable protein sources. Animal foods have no nutritious dietary fibre and also contain saturated fat and cholesterol that increase the risk of cardiovascular diseases. Major vegetable protein sources such as soy, peas and legumes have and require a variety of herbicides, pesticides and other measures detrimental to the environment to ensure high yields.

Oat is currently ranked seventh in total world grain production. Oat seed storage proteins have a favourable amino acid composition with high nutritional quality, functionality and health benefits. Oat seeds contain globulin, albumin, prolamin and glutelin proteins. Oat proteins have been identified as one of the most nutritional protein sources with sufficient essential amino acids.

Research efforts to improve yields and nutritional quality of oat proteins would be beneficial. If the protein content of oats could be increased from 12 to above 15%, the crop would be nearly ideal as a protein crop. It can be grown in an ecologically sustainable manner and provides nutritionally balanced proteins that could play an important role in meeting consumer needs for good, healthy and nutritionally balanced oat-based foods. It also lacks many of the diseases that attack wheat, peas, and oilseed crops. The escalating problems of disease, seen especially in pea and bean plants, do not occur in oats.

In this thesis, more than a thousand individual lines in a mutagenised oat population (TILLING population) were screened for total protein content using an elemental particle analyser (EPA). Using EPA, the total amount of nitrogen atoms in oat flour from the different lines tested was calculated according to Dr Dahl's (Dumas) method. This identified 230 lines with a seed protein content of 15% or higher. The protein content in the highest line was 24%. The 15 highest lines were selected for further studies. The protein content of individual seeds was determined. Crosses were performed between the six lines with the highest protein levels and the original non-mutated Belinda variety from which the mutagenised population was derived. The first filial generation (F1) crossbred seeds grown in the greenhouse and self-pollinated and individual seeds from the second filial generation (F2) offspring were analysed. This showed that the high-protein character was stably transferred

(inherited) to the next generation. The stability of the high protein content was also tested by propagation in the field followed by protein determination of the harvested material. Total dietary fibre (TDF), β -glucan and lipid levels were also measured in the selected lines. Results showed that the values for these components were not affected by the high protein content.

Amino acid composition was also analysed in several of the high-protein oats lines, and the essential amino acid content (EAAs) in these lines was evaluated according to FAO/WHO amino acid recommendations. Levels of individual AA in the different lines varied, but several of the high-protein lines contained sufficient levels for a normal portion of oat (85 grams or 3 ounces of cooked oatmeal) to cover daily intake. Consequently, these lines have sufficiently high total protein levels ($>20\%$) and a favourable amino acid composition, and have the potential to become a new source of vegetable proteins. This will promote the development of novel food products based on oat.

Soluble proteins were extracted from the five high-protein oat lines ($\geq 17\text{--}24\%$) and separated from protein aggregates using a flow-based technique known as asymmetric flow field-flow fractionation (AF4) in combination with online multi-angle light scattering (MALS) and (UV) detection. The relative percentages of soluble to total proteins were determined and compared with the original, non-mutated Belinda, and significant differences were shown, both between the lines and in relation to Belinda. The amino acid (AA) composition of isolated soluble proteins was analysed and little difference was found in amino acid composition between whole groats and protein extracts of high protein oat lines. If a dietary protein does not meet the nutritional requirement due to the lack of one or more amino acids, these amino acids are termed limiting amino acids. Lysine and threonine are the limiting amino acids in oats. These amino acids were found to be higher in freeze dried soluble protein extracts than in flour.

The avenin protein fractions of selected high-protein oat lines were also characterised using electrophoresis. From the different avenin protein groups that appeared in the gel, it was concluded that the introduced mutations did not change the avenins in the high-protein oat lines.

The effect of heat treatment on the soluble proteins of oat groats extracted using sodium phosphate buffer was analysed using AF4 together with online multi-angle light scattering (MALS) and (UV) detection systems. Heat-treating the oat groats resulted in a nearly 50 wt.% reduction in the soluble protein fraction, and the albumin and prolamin protein fractions appeared to be more affected than the globulin fraction.

In conclusion, all the lines with total protein levels $>20\%$ that were selected here also have a favourable amino acid composition. In addition, the levels of total

dietary fibre (TDF), β -glucans and lipids were similar to the levels normally seen in Belinda. This means that the high-protein character did not negatively influence the other macromolecules, i.e. their respective biochemical pathways do not overlap. The levels of nutritionally favourable globulin proteins were high in all the high-protein oat lines, so their amino acid composition was more favourable. The high-protein character was stably inherited and there were no differences in avenin protein content between the lines. We believe our results will be useful for breeders aiming to increase the content and nutritional value of oat protein. If successful, this would enable the development of novel feed and food products based on oat.

List of Papers

This PhD thesis is based on the following papers, referred in the text by their Roman numerals. The papers are appended at the end of the thesis

- Paper I Identification and Characterization of High-Protein Oat Lines from a Mutagenised Oat Population.
Bindu A. Sunilkumar, Svetlana Leonova, Rickard Öste, Olof Olsson
Submitted, Industrial Crops and Products
- Paper II Using Amino acid Analysis to assess Protein Quality in Characterization of High- Protein Oat lines from a Mutagenised oat population
Yi Ren, **Bindu A. Sunilkumar**, Rickard Öste, Olof Olsson
Manuscript
- Paper III Soluble protein characterization of High-Protein Oat Lines
Bindu A. Sunilkumar, Ana Rascon, Rickard Öste, Olof Olsson
Manuscript
- Paper IV Identification of discrepancies in grain quality and grain protein composition through avenin proteins of oat after an effort to increase protein content.
Bindu A. Sunilkumar, Eden Tareke
Agriculture & Food Security, Biomed Central
DOI 10.1186/s40066-016-0056-6
- Paper V The effect of heat treatment on the soluble protein content of oats.
J. Ray Runyon, **Bindu A. Sunilkumar**, Lars Nilsson, Ana Rascon, Björn Bergenståhl
Journal of Cereal Science, vol. 65, pp. 119-124, 2015.

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Other related publications by the author:

Paper VI A simple highly efficient method for protein extraction from cereal seeds especially suited for proteomic analysis.

Bindu A. Sunilkumar, Hans-Olof Johansson, Negar Kavooosi, Aakash Chawade, Lars Sjögren and Olof Olsson

Manuscript

Paper VII Review of analytical methods to measure oat proteins: the need for harmonized methods.

Bindu A. Sunilkumar, Eden Tareke

Submitted, Critical Reviews in Food Science and Nutrition

My contribution to the papers

Paper I - I was involved in study design. I identified the high-protein oat lines and performed the crossing and amplification in the greenhouse and data analysis. I performed the dietary fibre and β glucan analysis. I drafted all parts of manuscript with the help of the other authors.

Paper II – I was involved in study design, and helped to analyse the amino acids of high-protein oat lines and data analysis. I participated in drafting the manuscript with the other authors.

Paper III – I was the main designer of the study. I performed the flow fractionation experiment. I performed the data analysis and prepared the manuscript together with all co-authors.

Paper IV – I was the main designer of the study and experiments. I isolated the avenin protein and performed the protein quantification, characterisation and data analysis. I participated in writing the paper.

Paper V – I was involved in planning the study. I performed protein quantification, amino acid analysis and electrophoresis. I participated in flow fractionation studies, data analysis and writing the paper.

Abbreviations

TILLING	Targeting Induced Local Lesions in Genomes
TDF	Total dietary fibre
β- Glucan	Beta-glucan
EAAAs	Essential amino acids
EAAI	Essential amino acid index
AA	Amino acid
FAO	Food and Agriculture Organization
WHO	World Health Organization
FDA	Food and Drug Administration
EFSA	European Food Safety Authority
FIFFF	Flow Field-Flow Fractionation
AsFIFFF/ AF4	Asymmetrical Flow Field-Flow Fractionation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
MALS	Multi-angle light scattering
HPLC-UV	High Performance Liquid Chromatography- Ultraviolet
NSP	Non starch polysaccharide
E %	Energy %
GMO	Genetically modified organisms
DRI	Dietary reference intake
LDL	Low density lipoprotein
IAEA	International Atomic Energy Agency
EMS	Ethyl methanesulfonate
PCR	Polymerase chain reaction
HRM	High Resolution Melting
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
NGS	Next-generation sequencing
GC	Gas chromatography
GI	Glycaemic index
F2 generation	Second Filial generation
MW	Molecular weight

Thesis at a glance

Paper I

Aim: To identify high-protein oat lines from a population of EMS mutagenised oat lines.

Method: Using an elemental particle analyser (EPA), total nitrogen content was measured in 1050 oat lines. The best 15 lines, with protein content ranging from 17-24%, were identified. Protein content of individual seeds was measured. Specificity of high protein content was identified after amplification in the field. Segregation patterns were identified through back crossings to the parental cultivar. Total dietary fibre, β -glucan and lipid analysis were also carried out.

Evaluation methods: Total protein quantification (Dumas method for identification and selection of high-protein oat lines), SDS-PAGE (protein fractions), amplification in the field (stability), crossing and individual seed protein determination (segregation), dietary fibre, β -glucan and lipid analysis.

Conclusion: We show that oat lines with high levels of protein are present in the mutagenised population. The 31 mutant lines finally selected in this study will be particularly important in helping to meet consumer needs for high-protein oat products that are rich in β -glucan and lipid. Preliminary experiments show that approximately 70% of the selected lines also retained their high-protein character after cultivation in the field. These lines will provide an important gene source for breeders aiming to increase the content and nutritional value of oat protein.

Paper II

Aim: The aim was to assess the amino acid profiles of high-protein oat lines studied in paper I and to identify lines with a more balanced amino acid composition than the parental cultivar.

Method: The amino acid profiles for both essential and non-essential amino acids (except sulphur containing amino acid and tryptophan) were obtained by separating acid hydrolysed proteins on a Biochrom 31 amino acid analyser. In a separate experiment, cysteine and methionine were quantitatively oxidised to cysteine acid and methionine sulfone before injected into the amino acid analyser. Tryptophan was determined after papain-induced hydrolysis at alkaline pH adjustment and centrifugation, followed by a fluorometric analysis (Rickard, 1976).

Evaluation method: Amino acid analysis method based on ion exchange chromatography. Evaluation of essential amino acids (EAAs) according to the FAO/WHO recommendations.

Conclusion: Most of the high-protein lines contained a good balance of essential amino acids (EAAS), limited only in lysine, although the lysine amount varied between lines. A few lines contained such high amounts of lysine that the EAAI valued reached 97.36% of the reference pattern for children aged 3+ and adults.

Paper III

Aim: The aim was to analyse and compare the total amount of soluble proteins, the relative amount of monomeric proteins and protein aggregates and amino acid composition of soluble proteins in five high-protein oat lines and parental Belinda oat cultivar

Method: Asymmetric flow field-flow fractionation (AF4) together with online MALS and UV (280 nm) was used to quantify soluble proteins from the oat variety Belinda and from five high-protein oat lines identified from an oat-mutagenised population. Selected extracts of high-protein oat lines were analysed by amino acid analysis and SDS-PAGE.

Evaluation method: Asymmetric flow field-flow fractionation (AF4) together with online MALS and UV (280 nm) and amino acid analysis based on ion exchange chromatography and SDS-PAGE of oat extracts.

Conclusion: The results show that the soluble protein of all high-protein oat lines was twice the amount calculated for the control Belinda. All five high-protein oat lines had, from a nutritional point of view, a better amino acid composition than the parental cultivar Belinda.

Paper IV

Aim: To identify environmental effect like differences in geographical location, field, greenhouse, mutation and excess nitrogen as well as genetic effects on the total protein content of oat. Apart from total protein, another aim was to identify differences in avenin constitution of Belinda oat cultivated under varying conditions.

Methods: Quantification of total proteins and SDS-PAGE analysis of avenin electrophoretic patterns of 25 samples isolated from 20 different oat cultivars and 5 mutated oat lines grown under in different greenhouse and fields conditions.

Evaluation methods: Elemental particle analyser (Dumas method) for total protein quantification and SDS-PAGE analysis of avenin.

Conclusions: The results show that oats treated with nitrogen and the selected mutated lines showed increase in protein concentration, with consistent avenin loci to the parental line. This result provide information on the stability of modifications that will be useful for breeders aiming to increase the content and nutritional value of oat protein.

Paper V

Aim: The objective was to make use of the advantage of AF4 to evaluate the effect of heat treatment on total proteins from the commercially available oat variety SW Kerstin, and to evaluate heat induced changes in the aggregation patterns that may influence the utilisation of oat in more complex processes.

Method: The effect of heat treatment on the soluble protein content in oat groats was evaluated using asymmetric flow field-flow fractionation (AF4) in combination with online multi-angle light scattering (MALS) and (UV) detection. Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed the selective elimination of protein bands associated with the albumin and prolamins protein fractions because of heat treatment.

Evaluation method: Asymmetric flow field-flow fractionation (AF4) together with online MALS and UV (280 nm) and amino acid analysis based on ion exchange chromatography and SDS-PAGE of heat treated and non-heat treated oat extracts.

Conclusion: Heat treatment affects the solubility of oat proteins and may have a selective impact on the different soluble protein fractions in oats. Heat treatment could therefore influence the functionality of the soluble oat proteins and their uses in technical and/or functional food applications

Chapter 1. Introduction

The fundamental cause of failing health due to obesity and overweight is an increased intake of energy-dense foods that are high in fat and low in nutritious fibre. Obesity and overweight are defined as "abnormal or excessive fat accumulation that presents a risk to health". According to WHO, in 2014 more than 1.9 billion adults were overweight (WHO, 2014). Of these, over 600 million were obese. Easily accessible food and lack of exercise are typical reasons for this. Research shows that satiety is increased at higher protein intake (Westerterp-Plantenga, Lemmens, & Westerterp, 2012), which has led to the popularity of diets such as 'low carbohydrate high fat' (LCHF). This places greater demands on the food proteins, i.e. their amount, quality, source, and environmental sustainability.

Nordic Nutrition Recommendations (NNR) 2012 stipulate Recommended Intakes (RI) for protein as follows (Adamsson, Reumark, Cederholm, Vessby, Riserus, & Johansson, 2012). *Adults and children from 2 years of age: Protein should provide 10-20 % of the total energy intake (E%); Elderly (≥ 65 years): Protein should provide 15-20 E%, and with decreasing energy intake (below 8 MJ/d) the protein E% should be increased accordingly.* This intake of protein should adequately meet the requirements for essential amino acids (Rand, Pellett, & Young, 2003; Young & Pellett, 1994). For dietary planning purposes, the energy from protein should be 15% of total energy intake (15 E%), which corresponds to about 1.1 g protein per kg body weight per day. For elderly people, a suitable target for the amount of protein intake should be 18 E% (Campbell, Trappe, Wolfe, & Evans, 2001), which corresponds to about 1.2 g protein per kg body weight per day (Pedersen, Kondrup, & Børsheim, 2013; Rand, Pellett, & Young, 2003).

Consumption and production of protein-rich available food sources in today's market present many problems. Most proteins are derived from animal sources. However, but there are many good reasons to use vegetable proteins to a higher extent. Animal foods have no nutritional dietary fibre and also contain saturated fat and cholesterol, which increases the risk of cardiovascular diseases (Key, Allen, Spencer, & Travis, 2002; Lichtenstein, Appel, Brands, Carnethon, Daniels, Franch, et al., 2006). A high intake of red meat raises the risk of heart disease and certain cancers (Jankovic, Geelen, Streppel, de Groot, Kiefte-de Jong, Orfanos, et al., 2015; Key, Allen, Spencer, & Travis, 2002). The production of animal foods burdens the environment much more than the corresponding production of vegetables (Reijnders

& Soret, 2003), although growing protein-rich vegetable sources such as soybean, pea, and legume is not without agronomical problems either. Cultivating legumes is associated with various fungal diseases that reduce yield and burdens the environment. Cultivation of soy therefore requires a variety of pesticides herbicides and other measures with a negative impact on the environment (Pengue, 2005). In recent years, genetically modified soy, known as GMO soy, has completely dominated world cultures (Dros, 2004) due to their lower requirements of herbicides. However, since acceptance of GMOs is low, growing and selling such soy is also problematical, especially in Europe (Ho, 2014). Furthermore, soy has its own strong flavour and contains a variety of anti-nutrients such as lectins and saponins, which provide both a bitter taste and can cause various stomach problems and allergy (Cordle, 2004; Course, 1972; Garcia, Torre, Marina, & Laborda, 1997).

Oats (*Avena sativa* L.) are the cereal grains with the highest content of protein compared to other commonly used cereals such as wheat, corn, barley, rice and sorghum (Peter R Shewry & Nigel G Halford, 2002). The oat protein is of good quality, and oats contain the highest proportion of globular proteins of all cereals, with good bioavailability (Maruyama, Shands, Harper, & Sunde, 1975; D. M. Peterson, 1976; D. M. Peterson, Brinegar, & Webster, 1986; Robbins & Briggles, 1971). If the protein content of oats could be increased to > 15 E%, this crop would be nearly ideal, especially since it can be grown in an ecologically sustainable manner. It can also be a nutritionally balanced staple food and play an important part in meeting consumer needs for good, healthy, nutritionally balanced foods. Compared to other cereals and legumes, oats lack many of the diseases that attack wheat, peas and oilseed crops (Nasraoui, 2008; Nielsen, 2002).

Oats are particularly well suited to growing in Scandinavian conditions (Atterberg, 1887). The yield is very good and high quality seeds are produced. Oat is also an important rotation crop (F. Webster, 2011) and common cultivation problems seen in pea and bean plants do not apply to oats (Fuentes- contreras, Powell, Wadhams, Pickett, & Niemeyer, 1996; Papadopoulou, Melton, Leggett, Daniels, & Osbourn, 1999; Weibull, 1986). Oats are tasty and their protein completely lacks gluten, is less allergenic and has a favourable amino acid composition (Dissanayake, Truelove, & Whitehead, 1974; Garsed & Scott, 2007; C. G. Zarkadas, Yu, & Burrows, 1995).

The quantity and quality of oat protein is considered to be adequate for most uses, and little attention is currently paid to breeding new cultivars to increase protein concentration or improve amino acid composition (D. M. Peterson, 2011). However, we believe a lot of improvement can still be done and the objective of the work presented in this thesis was therefore to identify and characterise high-protein oat lines from an oat mutagenised population. Segregation patterns of high protein content, total dietary fibre, beta-glucan, and lipid content were evaluated. Amino

acid composition, solubility, inheritance of avenin and thermostability of protein characteristics were also studied as well as the stability of the high protein character in field grown plants.

Oats

Oats are an annual grass in the Gramineae grass family. The cultivated oat (*Avena sativa* L. and *Avena byzantiana* L.) can grow on every continent, but prefers temperate regions and thrives where summers are cool and wet, i.e. in areas such as northwest Europe and even Iceland (Bonsall, Macklin, Anderson, & Payton, 2002; Holopainen, Rickard, & Helama, 2012). Both *sativa* and *byzantine* types were introduced into North America in the sixteenth century (Coffman, 1977) but it is uncertain when more systematic oat cultivation and improvement started. It has been reported that the cultivated oats evolved much later than wheat. They were most likely grown as a mixture with wheat and barley in areas surrounding the north of the Mediterranean Sea during the third and second millennium BC (Jellen & Beard, 2000; Malzew, 1930; RajhathY & Thomas, 1972).

The genus *Avena* includes wild and cultivated species with polyploid series of diploids, tetraploids, and hexaploids and with a basic chromosome number of 7. Nineteen taxonomic species have been reported, of which ten are diploids ($2n = 14$) five are tetraploid ($2n = 28$) and four are hexaploids ($2n = 42$). The 19 taxa have been further divided into 11 genomic groups (Baum, 1977), each group representing a biological species, of which seven are diploid, three are tetraploid, and one is hexaploid. However, most of all the cultivated oats belong to the hexaploid group except *Avena strigosa* (S.) which is the only diploid oat (G. Ladizinsky, 1974; G. Ladizinsky & Zohary, 1971).

The acknowledged health benefits have made oats a unique and superior cereal that is a desirable ingredient in many food products. Compared to any other cereals, oats have the highest quality and quantity of protein (Maruyama, Shands, Harper, & Sunde, 1975; D. M. Peterson, 1976; C. G. Zarkadas, Yu, & Burrows, 1995). Other components are starch (Hoover & Vasanathan, 1992; Zhou, Robards, Glennie-Holmes, & Helliwell, 1998), lipids (D. Peterson, 2002; Vernon L Youngs & Webster, 1986; Zhou, Robards, Glennie-Holmes, & Helliwell, 1999) dietary fibre (H. Englyst, S. Bingham, S. Runswick, E. Collinson, & J. Cummings, 1989; H. N. Englyst, S. A. Bingham, S. A. Runswick, E. Collinson, & J. H. Cummings, 1989) and minor oat constituents, such as avenanthramides (C.-Y. O. Chen, Milbury, Collins, & Blumberg, 2007; H. N. Englyst, S. A. Bingham, S. A. Runswick, E. Collinson, & J. H. Cummings, 1989; D. M. Peterson, 2001), tocopherols (Handelman, Cao, Walter, Nightingale, Paul, Prior, et al., 1999; D. M. Peterson,

1995; Wojdyło & Oszmaiński, 2007), flavanones and glycosides (Pietta, 2000; Popovici & Weissenbock, 1976), which have distinct characteristics unique to oats.

Oats as a functional food for health

Functional foods are generally defined as *food that provide health benefits beyond basic nutrition when consumed on a regular basis at effective levels* (Hasler, Bloch, Thomson, Enrione, & Manning, 2004). Based on observational and clinical intervention studies, it is suggested that oats are effective in lowering blood cholesterol levels (de Luyken, & Pikaar, 1963; Judd & Truswell, 1981; Kerckhoffs, Hornstra, & Mensink, 2003; Malkki, Autio, Hanninen, Myllymaki, Pelkonen, Suortti, et al., 1992; Van Emden, Ball, & Rao, 1988). A daily intake of oat products that contain three grams of β -glucan, a soluble fibre, is estimated to reduce serum cholesterol by 5-15 % (Othman, Moghadasian, & Jones, 2011) and every 1% decrease in serum cholesterol would provide a 2-3% reduction in the observed rate of cardiovascular disease (J. Chen & Raymond, 2008). Numerous studies have also shown that consumption of whole grains containing a high amount of soluble fibre, such as oats and barley, are more effective in lowering blood cholesterol not only in humans but also in other animals (Beer, Arrigoni, & Amado, 1995; Bell, Goldman, Bistrrian, Arnold, Ostroff, & Forse, 1999; Jenkins, Kendall, Vuksan, Vidgen, Parker, Faulkner, et al., 2002; Onning, Wallmark, Persson, Akesson, Elmstahl, & Oste, 1999; Othman, Moghadasian, & Jones, 2011; Wood, 2004). Studies have also shown that diets with a low glycaemic index and high dietary fibre may reduce the risk of developing type-2 diabetes and other heart-related diseases (Ludwig, Pereira, Kroenke, Hilner, Van Horn, Slattery, et al., 1999; Meyer, Kushi, Jacobs, Slavin, Sellers, & Folsom, 2000; Salmerón, Ascherio, Rimm, Colditz, Spiegelman, Jenkins, et al., 1997; Wolk, Manson, Stampfer, Colditz, Hu, Speizer, et al., 1999). The U.S. Food and Drug Administration (FDA, 1997) has allowed a health claim for oat soluble fibres due to their ability to lower heart disease risk (FDA, 1997, 2003). The European Food Safety Authority (EFSA) has also approved the health claims associated with oat β -glucan due to its ability to reduce post-prandial glycaemic responses (Efsa Panel on Dietetic Products & Allergies, 2011). However, this ability depends upon the consumption of oatmeal or oat bran with optimum level (3 g per day) of soluble fibres over a long period of time (12 weeks) (Davy, Davy, Ho, Beske, Davrath, & Melby, 2002; Food & Drug Administration, 2002)

Several studies using mammalian tissue have shown that oat flavones and their glycosides have anti-proliferation and angiogenesis activity in human epithelial cells. In addition, oats have many other biologically active micronutrients beneficial to health, such as avenanthramides and cancer-preventive peptides like lunasin (64–

197 µg/g) in oats (H. N. Englyst, S. A. Bingham, S. A. Runswick, E. Collinson, & J. H. Cummings, 1989; Nakurte, Kirhnere, Namniece, Saleniece, Krigere, Mekss, et al., 2013; Robert W Welch & Yong, 1980). Oat avenanthramides are phenolic compounds that have an anti-inflammatory and anti-proliferative effect on several cancer cell lines (Meydani, 2006, 2007; Nie, Oishi, Doi, Shibata, & Kojima, 1997).

Consumption of the trace components in oats may alleviate the occurrence of several chronic diseases (Huang, lee, lee, lin, & lee, 2005; Manju, Balasubramaniyan, & Nalini, 2005; Nie, Oishi, Doi, Shibata, & Kojima, 1997; Osada, Imaoka, & Funae, 2004). In addition to antioxidants, oats also contain plant sterols known to lower cholesterol level significantly in humans if consumed in sufficient quantities (Ostlund, 2002). Oats also contain folate (495-604 ng/g cubic decimetre) which is the precursor for different forms of the B-vitamin family (Andersson, Lampi, Nyström, Piironen, Li, Ward, et al., 2008; Piironen, Edelmann, Kariluoto, & Bedő, 2008; Shewry, Piironen, Lampi, Nyström, Li, Rakszegi, et al., 2008). Pure, uncontaminated oats are safe for consumption by most individuals with celiac disease (Storsrud, Hulthen, & Lenner, 2003; Størsrud, Yman, & Lenner, 2003; Thompson, 2003). In view of the potential of oats to control a number of disease conditions, oats are considered as a multifunctional food (Robert W Welch & Yong, 1980).

Use of oat proteins as a feed ingredient

Oats are ideally suited for animals, especially non-ruminants, compared with barley and wheat because of their high fat (lipid) and protein with well-balanced amino acid composition. Less balancing is required with other amino acids compared to corn, wheat, and barley. Today, oats are often used as feed for animals, particular broiler chicken, turkey broiler, swine, horses, dairy and beef animals, lambs, laying hens, sheep, rabbits and pet foods (Cuddeford, 1995). If the oat feeds are supplemented with lysine, the resulting meal can increase weight, improve feed conversion and prevent excess carcass fat accumulation (Al Jassim, 2006). Groats containing 14-19% protein can supply most of the essential amino acids required by the horse (Rodiek & Stull, 2007). Unnecessary addition of oil to the formulated feed can be avoided if the groats contain 6-9% lipid (oil or fat) (Harris, 2009). Oats could also be used as the main source of concentrated energy and protein for all categories of rabbits, and does not need to be mixed with other source of energy and protein (F. Webster, 2016). In combination with a small amount of hay, oats can provide an inexpensive and very suitable food for ruminant animals.

Nutrient composition and nutritional quality of oats in comparison with other cereals

Although oats are recognised as an important source of high-quality fibre, lipid and protein, the increased availability of wheat and advances in technologies for milling and baking leaves oats behind wheat and other cereals in terms of production levels. Currently oat ranks sixth or seventh in the world cereal production, after rice, corn (maize), wheat, barley, sorghum and millets. The rank for usage in food is also lower than the above cereals (Robert W. Welch, 1995; R.W Welch, 2006). However, the nutritional profile and functionalities have made oats desirable ingredients for use in new food products (e.g. *Oatly* liquid oats). On average, oat seed kernel contains ~ 16% protein, 7% fat, ~10% dietary fibre (of which 4.5% β -glucan) and ~ 63% starch (Asp, Mattsson, & Onning, 1992).

Nutrient composition vs. nutritional quality

Nutrient composition can be determined by chemical or laboratory analysis. Nutrient quality is the ability of a foodstuff to satisfy dietary requirements. The dietary requirements and nutritional qualities may depend upon nutrient density, digestibility, and availability of nutrients of food or diets (Gilani, Cockell, & Sepehr, 2005). Nutrient composition can be changed during cereal processing, due to the depletion of fibre-rich bran layers along with associated vitamins and minerals, and the addition of fat, salt, sugar and other minerals for fortification. There are also variations in the nutrient composition of raw materials. These variations are due to genetic and environmental factors such as climate and fertility. In order to understand the nutritional quality of cereals, oat meal was compared with other whole grain cereals used for food or as food ingredients. These cereals are whole-grain common (bread) wheat (*Triticum aestivum*), cornmeal (*Zea mays*), brown rice (*Oryza sativa*), whole grain rye (*Secale cereale*), pearled barley (*Hordeum vulgare*), and sorghum (*Sorghum bicolor*).

Proximate constituents

The proximate constituents of food and feeds are water, ash (inorganic matter), fat (oil, lipid), protein, carbohydrate and fibre. The proximate component of oatmeal and samples of other whole grain cereals are shown in Table 1.1.

Table 1.1

Literature data on proximate constituents and energy values (representative values per 100g) for oats and other whole grains ^a

	Oatmeal	Whole-grain wheat	Cornmeal	Brown rice	Whole-grain rye	Pearled barley	Sorghum
Water, g	8.5	12.0	11.2	12.2	13.0	10.3	11.6
Carbohydrate, g	58.7	60.2	70.6	73.9	58.7	69.7	65.6
Protein, g	14.0	13.5	8.8	7.4	11.2	9.2	11.0
Fat, g	8.0	2.1	3.5	2.8	2.3	1.6	3.3
Dietary fibre, g	9.0	10.6	4.8	2.3	12.8	8.0	6.9
Ash, g	1.8	1.6	1.1	1.4	2.0	1.2	1.6
Energy, kJ	1,473	1,270	1,409	1,412	1,215	1,331	1,359
Energy, kcal	363	314	349	350	300	330	336

^a Compiled from data in U.S Department of Agriculture, Agricultural Research Service (2008) and Welch (2006). The table was formulated using the equation Carbohydrate = 100 (water + fat + proteins + fibre + ash) so that the total of the proximate constituents is 100%. Water makes up a relatively small proportion of all these cereals, and maintaining low levels is essential for storage stability.

As shown in Table 1.1, oatmeal and whole-grain wheat have the highest proportion of protein and a somewhat lower carbohydrate level compared with other cereals. Dietary fibre varies most between cereals, and is highest in oatmeal, rye, and wheat and lowest in rice. Ash content is relatively small in all cereals compared with other components. The dietary energy values reflect the amount of proteins, carbohydrates and fat present, but since the energy produced by fat is more than twice that of carbohydrates and protein, the higher fat content of oats leads to overall higher energy values compared with those of other cereals.

For an accurate examination of nutritional quality, a number of factors must be addressed. These include 1) the amount of each nutrient provided relative to the energy provided, 2) the potential contributions to nutrient and energy requirements, 3) the nutritional quality of fat and protein, and 4) the digestibility and availability of the nutrients.

Nutritional quality can be determined from Nutrient Density Scores. This is a ratio obtained by comparing the relative contributions of individual nutrients on an energy basis with the estimated requirements for that nutrient and for energy (Drewnowski, 2005). Table 1.2 shows the nutrient densities of oats and other whole grains for protein, fibre, and fatty acids.

Nutrient density = (Nutrient/100g) / (energy/100g)

DRI for nutrient/DRI for energy

DRI is the dietary reference intake.

Table 1.2Literature data on nutrient densities of oats and other whole grains for protein, fibre, and fatty acids ^a

	Oatmeal	Whole-grain wheat	Cornmeal	Brown rice	Whole-grain rye	Pearled barley	Sorghum
Protein	1.85	2.06	1.21	1.01	1.79	1.33	1.57
Dietary fibre	2.18	2.97	1.21	0.58	3.76	2.14	1.81
Linoleic acid	1.54	0.69	0.92	0.56	0.82	0.50	0.83
Linolenic acid	0.88	0.40	0.50	0.32	1.07	0.49	0.39

^a Calculated using data from Table 1.1 and 1.5 and from Food and Nutrition Board, Institute of Medicine (2004).

As can be seen from Table 1.2, oats provide a good source of protein (similar to wheat and rye), a good source of dietary fibre, and are a good source of linoleic acid and linolenic acid (Food and Nutrition Board, 2004).

Protein quality

The high nutritional quality of oat proteins depends on the high concentration of indispensable amino acids compared to other cereals, as shown in Table 1.3.

Table 1.3

Literature data on indispensable amino acid composition (g/100 g of protein) of the protein of oats and other whole grains in comparison with dietary requirements for adults and young children

Indispensable amino acid	Oatmeal	Whole-grain wheat	Corn meal	Brown rice	Whole-grain rye	Pearled barley	Sorghum	Dietary requirements ^{b,c}	
								Adults (> 18 y)	Young children (6-8y)
Histidine	2.1	2.3	2.6	2.4	2.2	2.1	2.2	1.7	1.9
Isoleucine	3.8	3.5	3.6	3.8	3.5	3.5	4.1	2.3	2.8
Leucine	7.2	6.7	11.1	8.2	6.2	6.7	14.6	5.2	6.6
Lysine	3.7	2.7	2.3	3.7	3.4	2.6	2.2	4.7	5.8
Cysteine	(2.7) ^d	(2.5)	(2.0)	(1.6)	(1.9)	(2.2)	(1.7)	(-)	(-)
Methionine	(1.8)	(1.2)	(1.6)	(2.1)	(1.4)	(1.6)	(1.4)	(-)	(-)
Cysteine + methionine	4.5	3.7	3.6	3.7	3.3	3.8	3.1	2.3 ^e	2.5
Phenylalanine	(5.0)	(4.6)	(4.4)	(4.8)	(4.5)	(5.1)	(5.0)	(-)	(-)
Tyrosine	(3.4)	(1.7)	(3.5)	(4.0)	(1.9)	(3.0)	(3.2)	(-)	(-)
Phenylalanine + tyrosine	8.4	6.3	7.9	8.8	6.4	8.1	8.2	4.1 ^d	6.3
Threonine	3.4	2.8	3.3	3.4	3.4	3.4	3.3	2.4	3.4
Tryptophan	1.3	1.5	0.7	1.3	1.1	1.6	1.1	0.6	1.1
Valine	5.1	4.3	4.0	5.8	4.8	5.0	5.4	2.9	3.5

^a Compiled from composition data in US Department of Agriculture, Agricultural Research Service (2008) and dietary requirements in Food and Nutrition Board, Institute of Medicine (2005)^b Grams per 100 g of protein, for adults over 18 years old (WHO, 2007)^c Grams per 100 g of protein, children (2-7) (is it 6-8 or 2-7 years ?, see table above (WHO, 2007)^d Values in parentheses are part of a combination.^e Requirements are combined

Table 1.3 shows that the protein of all cereals has adequate amount of all amino acids except lysine that is nutritionally limiting in all cereals (Maruyama, Shands, Harper, & Sunde, 1975). However, lysine is higher in oats and rice. The sulfur - containing amino acids such as cysteine and methionine are high in oats and rice. Tryptophan is only marginally sufficient in corn (Food and Nutrition Board, 2005; C. G. Zarkadas, Yu, & Burrows, 1995). The sum of the nine essential amino acids (histidine, isoleucine, leucine, lysine, methionine, cystine, phenylalanine, tyrosine, threonine, tryptophan and valine) in oats gave 44% of total protein, which is 10% higher than the recommended 34% for preschool children. Nutritional studies with young children and young women as subjects show that N balance is improved by supplementation of oat protein with lysine and or threonine (Maruyama, Shands, Harper, & Sunde, 1975). Oat proteins also show good digestibility (the degree to which macronutrients are digested and absorbed by the gastrointestinal tract) is about 90%. Rice and corn also have the similar percent of proteins digestibility (National Research Council 1989).

Dietary fibre components

Hipsley (1953) defines dietary fibre (DF) as “the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human intestine with complete or partial fermentation in the large intestine. It includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibre promotes beneficial physiological effects including laxation and/or blood cholesterol attenuation, and/or blood glucose attenuation”. Based on the solubility in water, DF is divided into insoluble and soluble fibre. Cellulose and some of the hemicellulose are insoluble in water (insoluble fibre) and whole gums and some of the hemicellulose are soluble in water (soluble fibre). Relatively small amounts of available energy can be produced from fibre with a total energy value of 8 KJ/g (Danish Food Composition Databank, 2008). Table 1.4 shows the total, soluble, and insoluble non-starch polysaccharides of oats and other whole grain cereals.

Table 1.4

Literature data on total, soluble, and insoluble non-starch polysaccharides (NSP), cellulose; and insoluble non-cellulosic polysaccharides, monosaccharide composition of total NSP in oats and other whole grains (per 100g, dry-matter basis)^a

	Oatmeal	Whole-grain wheat	Cornmeal	Brown rice	Whole-grain rye	Pearled barley
Total NSP, g	7.7	10.6	5.6	2.2	13.0	11.7
Soluble NSP, g	4.5	2.3	0.9	tb	4.5	3.9
(% total)	(58)	(22)	(16)	t	(35)	(33)
Insoluble NSP	3.2	8.3	4.7	2.2	8.5	7.8
(% total)	(42)	(78)	(84)	(100)	(65)	(67)
Cellulose, g	0.6	1.7	1.6	0.8	1.3	1.4
(% total)	(8)	(16)	(29)	(37)	(10)	(12)
Insoluble NSP	2.6	6.5	3.1	1.4	7.2	6.4
(% total)	(34)	(62)	(55)	(63)	(55)	(55)
Total NSP						
Glucose	65	24	34	42	26	49
Xylose	17	41	30	26	42	29
Arabinose	12	29	27	21	26	19
Galactose	2	3	5	5	2	1
Mannose	1	1	2	0	2	3
Uronic acids	3	2	2	5	2	1

a Data derived from Englyst (1989). All values are expressed on a dry matter basis.

b Trace

The total non-starch polysaccharide (NSP) and dietary fibre contents are highest in rye and lowest in brown rice and cornmeal. Oatmeal has highest level of soluble non-starch polysaccharides (4.5g/100g). In addition, the 58% of non-starch polysaccharides in oatmeal is soluble, which is high compared to all other cereals. However, insoluble non-starch polysaccharides and their components such as cellulose and insoluble non-cellulosic non-starch polysaccharides are less in oatmeal.

Glucose is the predominant monosaccharide of non-starch polysaccharides in oatmeal (65%), and is also high in barley (48%) and rye (42%). Xylose is the predominant monosaccharide in the non-starch polysaccharides of wheat and rye. Arabinose is also less in oatmeal compared with other cereals.

Beta-glucan

Oat β -glucan is a linear, unbranched polysaccharide composed of 30% 1-3 and 70% 1-4 linked- β D glucopyranosyl units. β -glucan is the major component of the soluble fibre fraction of oats, located mainly in the endosperm cell walls of oats and in the bran, more precisely in the aleurone and sub-aleurone layer Figure 1.1 (S. Miller, Wood, Pietrzak, & Fulcher, 1993; Skendi, Biliaderis, Lazaridou, & Izydorczyk,

2003; Wood, 2004, 2007). There is no β -glucan in the hull or in any other kernel tissues.

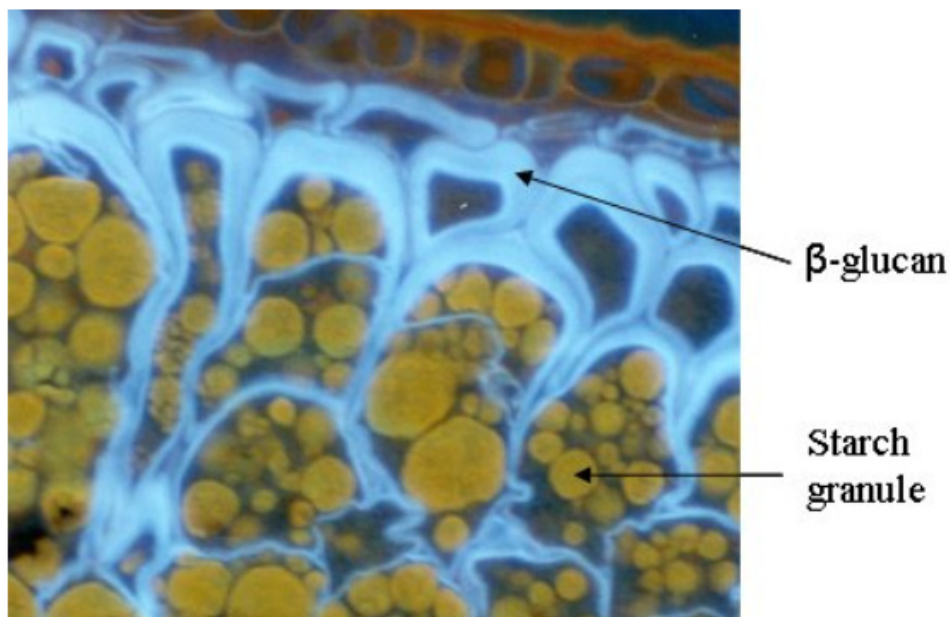


Figure 1. 1

Cross-section of oat bran layer stained with Calcofluor and Acid Fuchsin. Endosperm cell walls rich in β -glucan appear as blue and protein as brownish red. Courtesy of VTT Technical Research Centre of Finland

The β -glucan extracted from oats tends to have higher MW and is more readily soluble in water or dilute alkali compared to β -glucan from other cereals except barley (Bhatty, 1992; Johansen, Wood, & Knudsen, 1993; Li, Cui, & Kakuda, 2006). Several beneficial health effects are associated with oat β -glucan. It can lower elevated plasma cholesterol and reduce the risk of heart diseases, and this has been recognised in health claims (FDA, 1997).

Significant differences in the amount of β -glucan have been observed in cereals (Havrlentova & Kraic, 2006), but comparisons between cereals are difficult due to the inconsistencies observed in sample origin and lack of information about the sample preparation (D. M. Peterson, 2011). In oats and barley, much higher concentrations of soluble fibre were noticed than in other cereals. Oat and barley β -glucans are predominantly composed of β -(1-3)-linked cellotriosyl and cellotetraosyl units. However, barley β -glucan is more soluble and forms gels faster than oat β -glucan because of more cellotriosyl units and lower MWs (Lazaridou & Biliaderis, 2007). Gel characteristics are directly dependent upon the number of β

(1-3) linked cellotriosyl units, which are less in oat than in barley β -glucan (Rahar, Swami, Nagpal, Nagpal, & Singh, 2011). In oat bran, β -glucan content ranged from 5.5 to 9.0%, and in barley bran from 4.0 to 8.5%. In rye, the content is 1.5-2.5%, in wheat 0.5-1.5%, and in corn and rice only 0.5% (Wood, 2010).

Lipids

Oats are unique among the cereals in having high fat content, predominantly long-chain fatty acids that make up 95% of total lipids. Total lipids are rich in neutral lipids and polar lipids. The neutral lipids include triacyl glycerols, diacylglycerols and free fatty acids, and the polar lipids include phospholipids and glycolipids (de la Roche, Burrows, & McKenzie, 1977; Price & Parsons, 1975; Sahasrabudhe, 1979; V. Youngs, 1978). The total lipid content of the oat groat varies from 2% up to 13%, and is mainly located in oat kernels as oil bodies and as lipid bilayers. According to Banaś et al., 90% of the oat lipids are present in the endosperm, while the amount of lipid in the hull is relatively low and varies between 0.2 and 0.5% of hull weight (Banaś, Dahlqvist, Debski, Gummeson, & Stymne, 2000; Bryngelsson, Mannerstedt-Fogelfors, Kamal-Eldin, Andersson, & Dimberg, 2002). However, the hull can comprise 20-30% of the total weight of the seed, and some of the percentage of total lipid content depends on whether the sample is dehulled or non-dehulled (Brown & Craddock, 1972; Bryngelsson, Mannerstedt-Fogelfors, Kamal-Eldin, Andersson, & Dimberg, 2002; Krishnan, Reeves, Kephart, Thiex, & Calimente, 2000; Shewry, et al., 2008).

Synthesis and accumulation of the majority of lipids occurs in the early stages of grain development, varying between cultivars. In high-lipid varieties, up to twice the amount of lipid has been observed, and the accumulation continues until the grain attains maturity (Banaś, Dahlqvist, Debski, Gummeson, & Stymne, 2000). Other significant lipid components are phospholipids, glycolipids and minor amounts of sterols and tocopherols (Robert W Welch, 1975; Robert W Welch & Lloyd, 1989). The fatty acid composition of oat in comparison with other cereals is shown in Table 1.5.

Table 1.5

Literature data on fatty acid composition (representative values, g/100g of total fatty acids) of oats and other whole grains ^{a,b}

	Oatmeal	Whole-grain wheat	Cornmeal	Brown rice	Whole-grain rye	Pearled barley	Sorghum
Palmitic (16:0)	9	18	12	22	15	22	13
Stearic (18:0)	2	2	2	2	1	1	2
Oleic (18:1)	36	18	32	34	17	13	34
Linoleic (18:2)	38	56	50	38	58	56	46
Linolenic (18:3)	2	3	2	2	7	5	2

^a Compiled from data in Welch (1995a, 2006)

^b Total includes 2-3% of trace fatty acids

Polyunsaturated linoleic acid is the major fatty acids in all cereals, and oleic acid and palmitic acid are the other two. Polyunsaturated linoleic and linoleic acids are the fatty acids in oats that are of nutritional importance. Oat lipids are highly heritable in terms of both concentration and fatty acid composition. Lipid concentration is strongly influenced by grain weight, protein content, β -glucan, and starch content (Baker & McKenzie, 1972; Heneen, Banas, Leonova, Carlsson, Marttila, Debski, et al., 2009). Studies report discrepancies in the values for total lipid content and other macromolecules of oat. Zhu et al. (2004) reported that increase in protein content of oat does not affect the amount of lipid accumulation in the endosperm (Zhu, Rossnagel, & Kaeppler, 2004).

Micronutrient composition

Minerals and vitamins are micronutrients. Minerals form the inorganic (or ash) components and vitamins are the minor organic components. Both serve as the enzyme co-factors or their precursors, and are mainly concentrated in the bran layer.

As shown in Table 1.6 the major minerals in oats are phosphorous and potassium, with smaller amounts of magnesium and calcium. The concentrations of these major minerals are relatively high in oats, in comparison with other cereals. Even the minor minerals, such as iron, zinc, and manganese, are present in oats at levels that are generally higher than in other cereals. Oats also appear to be a good source of calcium, magnesium, iron, zinc, manganese, copper and phosphorous.

Table 1.6

Literature data on the mineral content (representative values. Mg/100 g of fresh weight) of oats and other whole grains^{a, b}

	Oatmeal	Whole-grain wheat	Cornmeal	Brown rice	Whole-grain rye	Pearled barley	Sorghum
Potassium	389	373	319	247	337	286	318
Phosphorus	459	333	266	302	367	242	289
Magnesium	145	129	134	127	107	80	156
Calcium	54	36	12	22	32	24	28
Sodium	9	4	38	4	3	5	15
Iron	4.3	3.9	3.2	1.6	2.7	2.7	4.8
Zinc	3.4	2.9	1.9	1.9	3.4	2.1	2.2
Manganese	4.1	3.5	0.6	3.0	1.7	1.2	1.8
Copper	0.44	0.42	0.30	0.56	0.44	0.39	0.98

^a Compiled from data in U.S Department of Agriculture, Agricultural Research Service (2008) and Welch (2006).

^b Water content as in Table 1.1

Oats are a particularly good source of vitamins, such as pantothenic acid, thiamine, riboflavin, folic acid and biotin. Levels of vitamin E, pantothenic acid, riboflavin and folic acids are relatively high compared with wheat, barley, rye and rice. Niacin and vitamin B6 are low in oats, but high in wheat and barley (Welch 2006, U.S Department of Agriculture 2008).

To conclude, the nutritional importance of oat is unique and is superior to any other cereals. This is due to high content of fat and protein, and comparatively high levels of essential amino acids. Oats have high content of dietary fibre and soluble fibre. Oat β -glucan is readily soluble in water and has a high molecular weight. Oat β -glucan can reduce elevated cholesterol level and thereby the risk of heart diseases. Avenantramides are phenolic acids with antioxidant properties and beneficial functions. Compared to other temperate cereals such as wheat, barley, rye and sorghum, oats have various advantages. They provide high-quality proteins and fibre, but lack many of the diseases that attack wheat, barley, peas and oilseed crops. This also makes it an important rotation crop, where the crops in the crop rotation grown after oats have higher yields. The growth problem observed in pea and bean plants does not occur in oats. Oats are tasty, the protein completely lacks gluten, and oats are less allergenic and are safe for most celiac patients.

Chapter 2. Oat storage proteins

The storage proteins of oats are of particular importance because they determine not only the total protein content of the seed but also contribute to the nutritional quality of food for humans and feed for livestock. The oat storage proteins also have several functional properties relevant in food processing (P. R. Shewry & N. G. Halford, 2002). The protein content of oats is high compared to other cereals and ranges from 11-20% (D. M. Peterson, 1992). A protein content of up to 24% has also been reported (Doehlert & McMullen, 2000).

Storage proteins are mainly located within protein bodies in the starchy endosperm and aleurone layer. The distribution of total proteins in the oat kernels is the same as other cereal grains, and are found in embryo (30%), starchy endosperm (10%) and in the bran (20%). Oat hull accounts for only 2% of total protein (Y. V. Wu, Sexson, Cavins, & Inglett, 1972). Concentration of oat proteins varies considerably among cultivars, species, and cultivars grown in different environments and at different fertility levels (D. M. Peterson, 1976). During seed germination, most of the proteins are proteolytically degraded and used for other protein synthesis in root and shoot development (Shutov & Vaintraub, 1987).

Classification and solubility fractionation

Oat storage protein is characterised by different extraction procedures using water, salt solution, alcoholic solution and dilute alkali or acid (Osborne 1924). The different solubility fractions are classified into four Osborne groups. The predominant oat protein fraction is globulin (soluble in high salt concentration). Other fractions are albumins (water soluble), prolamins (soluble in dilute aqueous ethanol) and glutelins (soluble in dilute acids or alkalies). A combination of several extraction procedures and SDS-PAGE analysis showed that 70-80% of protein bands in the total protein were found with a globulin fraction. Albumin comprises 9-20% of total protein (D. M. Peterson, Brinegar, & Webster, 1986; L. S. Robert, Nozzolillo, Cudjoe, & Altosaar, 1983). The least prevalent prolamins comprise only about 12% of the total protein, which is unique to oat compared to other cereals (S. Kim, Saur, & Mossé, 1979; D. M. Peterson, 1976). The distribution of oat proteins

and its characterisation is quite different when compared with other cereal grains because the soluble fraction of oat proteins is distributed in endosperm as storage proteins. In other cereals, for example wheat, insoluble endosperm proteins are practically equal to the storage proteins (Triboï, Martre, & Triboï-Blondel, 2003).

Globulin

The oat globulin was first reported by Danielson (1949) using analytical centrifugation. Later the hexameric nature of globulin was proposed by Peterson (1978) using sedimentation equilibrium analysis. The analysis showed that the native globulin located in the endosperm has a sedimentation coefficient of 12.1 with a total molecular weight of 322,000 daltons. Globulin has two polypeptide subunits joined by disulphide bonds. They are α (larger) and β (smaller) subunits with molecular weight of 32,000 daltons and 22, 000 daltons respectively. The overall molecular weight of the unreduced $(\alpha\beta)_6$ subunit is $\sim 53,000$ -58,000 daltons (Matlashewski, Adeli, Altosaar, Shewry, & Mifflin, 1982).

Several studies have shown that the increase in total oat protein is primarily dependent on the increase in the globulin fraction (D. M. Peterson & Smith, 1976; Portch, MacKenzie, & Stepler, 1968; Völker, 1975). In the developing oat seeds, the ratio of globulin increases as a percentage of total protein increase, which is controlled by gene translation. There is a linear increase in the synthesis of globulin from 2 days of anthesis up to 21 days after anthesis (Colyer & Luthe, 1984). Synthesis of α and β bands polypeptides occurs at an early stage, six to eight days after anthesis, as precursor on the polysoms of the rough endoplasmic reticulum. Later they are transported into the developing protein bodies and cleaved to corresponding polypeptide subunits (Adeli & Altosaar, 1983; Saigo, Peterson, & Holy, 1983). The proportion of protein concentration can vary according to N fertiliser, genotype, growing location and environment (Eppendorfer, 1977; D. M. Peterson & Smith, 1976; Völker, 1975).

Avenin

Avenin, the oat prolamin, is the least prevalent protein fraction of oat that contains several polymorphic components. They are heterogeneous proteins containing several groups that differ in size and charge (L. S. Robert, Nozzolillo, Cudjoe, & Altosaar, 1983). Three groups of oat avenin have been identified using starch gel electrophoresis. They are called α , β and γ avenins, but β avenins are present only in trace amounts (S. Kim, Saur, & Mossé, 1979). Intra- and interspecific heterogeneity in avenin protein fractions has been observed in diploid, tetraploid and hexaploid *Avena* species (S. Kim, Saur, & Mossé, 1979; Lookhart, 1985; L.

Robert, Nozzolillo, & Altosaar, 1983; Souza & Sorrells, 1990). Avenin is one of the suitable protein candidates for determining species relationships and oat cultivar identification (S. Kim, Saur, & Mossé, 1979; Souza & Sorrells, 1990). Synthesis of avenin starts from four to six days after anthesis and continues up to seed maturity (Chestnut et al 1989).

Albumins and glutelins

Most of the metabolically active proteins of oat are the water soluble albumin fractions, which contain enzymes that account for 9-20 % of total proteins. Recent analysis of oat proteins using asymmetric flow field-flow fractionation (AF4) shows that oat albumin fraction mainly comprises monomeric proteins (Runyon, Nilsson, Alftren, & Bergenstahl, 2013). Oat glutelins are protein fractions known as 'residual proteins', and extraction of the fractions is usually incomplete (Landry & Moureaux, 1994). Glutelins, soluble in dilute acids or alkaline accounted for 21-27% of total protein (D. M. Peterson, Brinegar, & Webster, 1986). The quantity of its extraction depends directly on the strength of extraction methods used for the separation of other oat protein fractions such as albumin, globulin and prolamins. There are similarities in composition between oat globulin and glutelins. Unextracted globulin will be recovered in the glutelin fraction, leading to discrepancies in the proportion of globulin content (D. M. Peterson, 1976; D. M. Peterson, Brinegar, & Webster, 1986; L. S. Robert, Nozzolillo, Cudjoe, & Altosaar, 1983).

Amino acid composition

The amino acid composition of oats is distinct among cereals and is close to the indispensable amino acid requirements for adults (FAO, 1990 (Table 7). However, lysine levels are nutritionally limiting in oats. Threonine can sometimes be a second amino acid that is limited.

Different Osborne solubility fractions such as albumin, globulin, avenin and glutelins have distinct amino acid compositions (Draper, 1973; Y. V. Wu, Sexson, Cavins, & Inglett, 1972). Globulin has a higher proportion of basic amino acids (lysine, histidine and arginine) and asparagine-aspartic acid. Albumins also have higher levels of lysine, asparagine, aspartic acid and alanine. However, the amount of these amino acids in albumin is lower than in globulin (Draper, 1973). Oat avenin has a high level of glutamine-glutamic acid. If the proportion of solubility fractions changes as a result of agronomic or other factors, the amino acid content of total oat protein will change accordingly. In most cereals, except oat and rice, prolamins fraction is the most predominant protein fraction. Oats and rice are high in globulins

and low in prolamins, so the protein quality of these two cereals is superior to other cereals.

Robbins et al. (1971) surveyed 289 oat samples representing oat cultivars obtained from and Canada between 1900 and the 1970s (Robbins & Briggles, 1971). This is one of the most comprehensive reports available for comparing cultivars through various oat breeding programmes. According to the authors, the smallest variability among amino acids was found for threonine and lysine, which averaged 3.3% and 4.2% of protein (N x 6.25). These figures are lower than the FAO reference standard values for threonine (4.2%) and lysine (5.5%) (FAO/WHO, 1991).

The amino acid composition of oat groat tissues such as embryonic tissue (embryonic axis and scutellum), bran (aleurone layer) and starchy endosperm and in the embryo was also reported. The embryonic tissue has higher lysine and glutamine/glutamate concentrations (Pomeranz, Youngs, & Robbins, 1973). The embryonic axis and scutellum have similar compositions, except that bran has a higher proline level. The embryo has very little effect on total amino acid composition (Vernon L. Youngs & Peterson, 1973).

Chapter 3. Application of oat proteins in industrial processes

The physical and chemical properties of proteins and their application in food processing are a major focus among the scientific community, consumers and food manufacturers. Apart from their nutritional value, proteins play important roles in the expression of sensory and organoleptic attributes of foods. Several of these characteristics depend upon various biophysical attributes of proteins, and are often affected by the structural changes that can occur when food components undergo different types of processing (Kinsella & Shetty, 1979). The extent of how functional properties change in the constituent proteins depends ultimately on their size and specific amino acid sequences and how they interact with other macromolecules during the processing steps (Nakai, 1983).

To understand the applicability and suitability of food systems, it is important to obtain proper knowledge of the functionality of the particular proteins present in each food system, since properties like solubility, emulsification and foaming can vary between food systems and type of processing (Cooper, Vaclavik, & Christian, 2003; Kinsella & Shetty, 1979). In Table 3.1, typical functional properties of proteins in different food systems are shown. However, these functionalities can be altered by several modification procedures (Panyam & Kilara, 1996), either physical, chemical or biological.

Table 3.1

Literature data on functional properties of proteins in different food systems

Functional property	Mode of action	Food system
Solubility	Protein solvation	Beverages
Water absorption and binding	Hydrogen bonding of water Entrapment of water (no drip)	Meat, sausages Breads, cakes
Viscosity	Thickening; water binding	Soups, gravies
Gelation	Protein matrix formation and setting	Meats, curds, cheese
Cohesion-adhesion	Protein acts as adhesive material	Meats, sausages, baked goods, pasta
Elasticity	Hydrophobic binding in gluten Disulphide links in gels	Meats, bakery
Emulsification	Formation and stabilisation of fat emulsions	Sausages, bologna, soups, cakes
Fat absorption	Binding of free fat	Meats, sausages, doughnuts
Flavour-binding	Adsorption, entrapment, release	Simulated meats, bakery etc.
Foaming	Form stable film to entrap gas	Whipped toppings, chiffon desserts, angel cakes

Utility of oat protein functionality

Thermal stability

Chemical modifications of oat proteins can improve their solubility (Mirmoghtadaie, Kadivar, & Shahedi, 2009; Yong, Yamaguchi, Gu, Mori, & Matsumura, 2004), emulsifying properties (C-Y Ma & Wood, 1987; Ponnampalam, Goulet, Amiot, Chamberland, & Brisson, 1988), water hydration capacity (Ching-Yung Ma, 1984), fat binding capacity (Mirmoghtadaie, Kadivar, & Shahedi, 2009) and foaming capacity and stability (Mohamed, Biresaw, Xu, Hojilla-Evangelista, & Rayas-Duarte, 2009). Oat proteins have a potential to be used as emulsion stabilisers, edible coatings and as functional additives in food systems (C.-Y Ma & Harwalkar, 1987; C-Y Ma & Wood, 1987).

Oat globulin preparation has high thermal stability with a denaturation temperature of about 110°C (Harwalkar & MA, 1987; Ching-Yung Ma & Khanzada, 1987). At even higher temperatures, dissociation of polypeptide chain occurs that initially results in the formation of soluble aggregates and, under more severe conditions, insoluble aggregates are formed that provide a higher thermal stability at 100 or 110°C (C.-Y Ma & Harwalkar, 1987). Consequently, oat globulin preparations can be used as ingredients in food where a high thermal stability is needed during the heat processing steps.

Gelling/coagulation

Gelling property is an inherent characteristic of globular proteins and is one of the most important functional properties of foods that undergo processing (Doi, 1993; Ziegler & Foegeding, 1990). Oat globulins can form gels with a smooth texture if heated at temperatures below the denaturation temperature, and oat globulin gelation at alkaline pH can be used to replace dairy and egg proteins in vegetable food formulations (Ching Yung Ma, 1983).

Oat globulin → Protein denaturation → Gel / coagulum formation
(Temp: below denaturation at pH10)

Flow properties

Flow properties of oat globulin dispersion are important for food manufacturers in the production of protein products. They not only improve the mouth feel but also improve the textural quality of finished food products. Such a pseudo-elastic flow behaviour of oat globulin is noticed during deamidation, acylation, and trypsin hydrolysis and linoleate treatment (C.-Y Ma, 1993). Consequently, oat globulins have a potential use as quality control of both the manufacturing process (change in thermomechanical properties during processing) and also for textural quality of final protein based food products.

Protein concentrates

Oat protein concentrates obtained through both wet and dry milling process have a good amino acid composition, a bland flavour, and useful functional properties such as hydration capacity, emulsion activity and emulsion stability. They are suitable as nutritional, bland and functional additives in food systems. Oat protein isolates can also be used as fortifiers to increase the organoleptic properties of neutral and acidic beverages (Cluskey, Wu, Inglett, & Wall, 1976) and as additives to wheat flour to increase the taste and nutritional quality of bread (D. A. Youngs, 1978). Chemically modified oat proteins have been produced with increased solubility, emulsifying properties, fat binding capacity and water hydration capacity exceeding wheat gluten, and with fat finding capacities significantly higher than both soy gluten and soy protein (C.Y Ma, 1983). In many respects, oat proteins are comparable to, or even superior to, soy protein isolates (Y. Wu, Cluskey, Wall, & Inglett, 1973).

Though oat globulin preparations have high thermal stability and have several advantages when used as a food ingredient, nutritional value can be lost by heat treatment above a critical level ($>110^{\circ}\text{C}$). Heat can denature the proteins, leading to

protein unfolding, often followed by a decrease in solubility of globulin due to protein aggregation (Privalov & Makhatadze, 1990). Excessive heat can also lead to chemical alterations of amino acid residues like dehydration of serine or deamidation of glutamine and asparagine, and to destruction and biological inactivation (blocking) of amino acids (Finot, Bricout, Viani, & Mauron, 1968). These changes further reduce the nutritional and functional properties of proteins.

This negative effect on nutritional value and bioavailability is even more pronounced when the heat treatment limits essential amino acids (Ajandouz & Puigserver, 1999). In the presence of reducing sugars, high temperature treatment can lead to Maillard reactions. It is a chemical reaction between an amino acid and a reducing sugar, usually requiring the addition of heat (Namiki, 1988). The amino acid lysine is particularly susceptible to such reactions and consequently to decrease of lysine, one of the most limiting amino acids in cereals (Nursten, 2005; Parker, Hassell, Mottram, & Guy, 2000; Tang, Wu, Le, & Shi, 2012). Other amino acids such as arginine, tryptophan, cysteine and histidine may also be modified, which negatively affects the final nutritional value (Hurrell, 1990). Apart from amino acid destruction, the heat treatment can provoke a decrease in amino acid bioavailability. The formation of cross links between peptide chains leads to blocking of amino acid side chains lysine and arginine, and reduces digestibility. Most of the reactions are irreversible (E. Miller, Carpenter, & Milner, 1965). These changes reduce protein efficiency ratio and net protein utilisation.

Chapter 4. The mutagenised oat population

There are several examples of quantitative and qualitative alterations of seed storage proteins, carbohydrates and other specific food components that emanate from various mutagenesis programmes initiated already before World War II. The International Atomic Energy Agency (IAEA) in Vienna and FAO in the U.S. have documentation of several thousand varieties that contain induced mutations. Special emphasis was placed on increasing protein levels in food due to a lack of protein-rich food. Since 70% of the per capita supply of proteins in the world is derived from plants, the ambition to increase the protein content was especially relevant (Hoffman & Falvo, 2004; Young & Pellett, 1994). The aim of many research programmes was to find ways to increase the world's total protein production by means of mutation and selection for plants with increased protein levels (Dannenhoffer, Bostwick, Or, & Larkins, 1995; Gottschalk & Wolff, 2012; Kaul, 1973; Munck & Shewry, 1992; Parveen, 2015). Consequently, mutation-bred plants have a history of being safe and do not cause ill health (Ahloowalia, Maluszynski, & Nichterlein, 2004; Maluszynski & Szarejko, 2003).

Oat breeding has been going on for at least 200 years (Clifford, 1995; Coffman, 1977; Holland, 1997; Stuthman, 1995; F. H. Webster, 1996; R. Welch, 2012). The first cultivated oats were probably hull-less and consumed without cooking or processing. The first documented crosses in oats was by Shirreff (1873). After the rediscovery of Mendel's work and the appreciation of Johanson's Pure Line Theory (continuous selection of pure lines will not lead to further improvements) breeders started to understand the importance of variation in the breeding populations and why hybridisation and mutation would increase the probability of creating new traits.

Mutation breeding

Chemical agents and radiation have been used in breeding programmes since the 1930s to generate genetic variability. To date, a total of 3234 cultivars have been produced by different mutation breeding technologies. Currently, 23 different oat mutant varieties are registered (<http://mvgs.iaea.org/Default.aspx>).

Targeting Induced Local Lesions in Genomes (TILLING) is a platform developed for cereals that combines classical chemical mutagenesis with various PCR-based screening methods to identify mutations in a given gene of interest (McCallum, Comai, Greene, & Henikoff, 2000). Two chemicals in particular have been commonly used to induce mutation, sodium azide (NaN_3) and ethyl methane sulphonate (EMS). Both are alkylating nucleotides that introduce point mutations randomly distributed over the entire genome.

EMS usually causes G/C to A/T transitions, leading to mutations that can be nonsense, missense and silent (Drake & Baltz, 1976; het Veld, Zdzienicka, Vrieling, Lohman, & van Zeeland, 1994). Nonsense mutations are more detrimental than the other two because, by introducing a stop codon, the translated protein is truncated and often loses its biological activity. Missense mutation will alter one amino acid (AA) in a protein chain, and its biological effect depends on whether the AA change will alter the activity of the corresponding protein. If the mutation occurs in a catalytic domain or affects protein folding and assembly, the functionality of the protein could be affected, depending on the nature of the replacing AA. Silent mutations have no effect on the protein sequence or functionality, since they either cause a neutral AA replacement or affect the wobbling base in such a way that no AA exchange occurs.

When producing a mutagenised population, it is important to introduce as many mutations as possible per genome in order to increase the variation as much as possible without killing the plant. In chemical mutagenesis of cereals, the target survival rate is usually 30-80% (Mba, Afza, Bado, & Jain, 2010). After mutagenesis, the resulting M1 seeds are planted and allowed to self-fertilise and produce a new generation of seeds (M2). Each batch of M2 seeds will make up a specific line (Chawade, Sikora, Brautigam, Larsson, Vivekanand, Nakash, et al., 2010). The more lines the better, since each line carries a set of unique mutations. Recent experiments have shown a strong correlation between the ploidy level in the plant mutagenised and the number of mutations that can be introduced (Anderson, Sirjusingh, & Ricker, 2004; Chawade, et al., 2010; Gerstein, 2013; Kondrashov & Crow, 1991). For hexaploid plants such as oat and wheat, the mutation frequency can be as high as one mutation per 25kb. The advantage of this is that fewer lines are then required in the total population to cover all genes. In diploid plants such as rice and barley mutation, frequency is much less, so bigger populations are required.

Identification of novel traits in mutated populations

In the oat mutagenised population used here (Chawade, et al., 2010), traits like differences in leaf shape, colour, presence of pubescence on the leaves or stem could be observed, although most lines (approximately 95%) appeared phenotypically normal. However, when growing the population in the field, other phenotypes might occur due to various environmental conditions. Environmental effects will depend on the particular mutant combination present in each line and on the strength of the genetic factor for a specific phenotype or trait. If genetic factors behind a particular trait are strong, the variation in the expression of that trait caused by the environment is expected to be less.

After the development of a mutagenised population, different genotypic and phenotypic screening methods can be used to identify individual lines in the population that carry a specific mutation or phenotypic trait. The major genotypic methods presently used are Next Generation Sequencing (NGS), Li-Cor, HPLC, electrophoresis, capillary electrophoresis, High Resolution Melt (HRM) technologies and MALDI-TOF separation of fragmented PCR amplified regions (Mardis, 2008; McCallum, Comai, Greene, & Henikoff, 2000; Till, Reynolds, Greene, Codomo, Enns, Johnson, et al., 2003; Uauy, Paraiso, Colasuonno, Tran, Tsai, Berardi, et al., 2009; Xin, Wang, Barkley, Burow, Franks, Pederson, et al., 2008) Chawade et al., 2010; Sikora et al, 2011).

Alternatively, various biochemical/analytical methods can be used to directly identify lines with altered macromolecule levels, like high fat, starch, protein, fibre composition or reduced levels of toxic compounds. More specific biochemical reactions can also be addressed; all that is needed is a specific assay sensitive enough to detect effects of single dominating mutations (Sikora et al, 2011). Once a specific trait has been identified in the mutagenised population, tests to confirm the character should be performed. Especially good lines can also be tested for stability in the field, crossed to verify the genetic inheritability of the character and then introgressed into elite varieties lacking the character. Once the specific mutation behind a trait has been identified, molecular markers for the trait can be developed. That will greatly enhance the outcrossing efficiency, and other random mutations that have nothing to do with the trait can be eliminated.

To conclude, a combined biochemical and TILLING technological platform makes it possible to identify useful new genetic variation in ergonomically and sociologically important crops, thereby providing unique possibilities to identify and develop novel traits in crops with excellent agronomic properties.

Chapter 5. Present work

Overall aim

The overall aim of this thesis was to identify and develop oat lines with increased protein levels and enhanced amino acid concentration of nutritionally limiting amino acids as compared to the existing cultivars.

Specific aims

To identify and characterise

- High-protein oat lines from a TILLING oat population and to understand the inheritance and stability of the identified high protein character by various crossings. To investigate whether an increase in protein content leads to a change in other macromolecules like total dietary fibre (TDF), β -glucans and lipid content in the best lines (Paper I).
- Amino acid composition of high-protein oat lines; more specifically to calculate the variability among high-protein oat lines for the nutritionally limiting amino acids and to compare the results with dietary recommendations (Paper II).
- Total as well as soluble protein percentage, proportion of solubility fraction and the amino acid composition of soluble extracts of high-protein oat lines. To examine whether the increased grain protein in high protein oat lines is due to increase in the proportion of globulin or other fractions (Paper III).
- The avenin proteins of high-protein oat lines. Avenin proteins of mutated oat lines were compared with parental cultivars cultivated under different field- or greenhouse conditions during different years (Paper IV).
- Effect of heat treatment on soluble proteins in the commercial variety Kerstin. More specifically, to understand the effect of heating in the protein fractions and their amino acid composition (Paper V).

Methodology

Table 5.1
Design and outcome

Papers	Design	Outcome
I	To quantify the total protein percentage and individual seed protein content, of individual seeds of TILLING oat lines using EPA.	More than 1000 mutated oat lines were screened. Fifteen lines were selected with total protein ranges from 17-24%. Individual seed analysis, stability of high protein content in the field were also tested.
II	To analyse the amino acid composition of selected high protein oat lines using hydrolysis method	Amino acid analysis of 31 high protein oat lines using hydrolysis and fluorometric assay. Lines with high amounts of favourable amino acid composition were identified
III	Total and soluble protein quantification of selected high protein oat lines using AF4 analysis	Total soluble proteins were quantified for 5 high protein oat lines and Belinda. Relative soluble protein percentages were also quantified
IV	Discrepancies in avenin protein fractions were identified using SDS-PAGE	Different pattern of avenin allele in electrophoretic gel allowed the comparison within and between groups. This analysis shows that the cultivars obtained from Swedish fields differed in the number of alleles compared with the parental cultivar, so were not stable across sites
V	Effect of heat treatment on soluble protein content using AF4 and amino acid analysis	Reduction in total and soluble protein, essential amino acid components were identified

Instruments used were the Elemental Particle Analyser for protein quantification, the Amino Acid Analyser for amino acid analysis, and the Asymmetrical flow field-flow fractionation (AsFIFFF or AF4).

Protein quantification using Elemental Particle Analyser (EPA)

The Elemental Particle Analyser (EPA) was used to quantify proteins of TILLING oat lines (Paper I), soluble protein of selected high- protein oat lines (Paper II) nitrogen-treated Belinda and Belinda grown under different geographical conditions (Paper IV) and normal, as well as heat-treated Kerstin oats (Paper V).

The elemental analyser, also known as the Dumas Nitrogen Analyser, is an automated system operating according to the dynamic flash combustion method. Unlike the Kjeldahl method, EPA needs no toxic chemicals, so it is an

environmental friendly method (Strickland & Parsons, 1972) accepted as an AOAC method (Cunniff & Association of Official Analytical, 1995). The principle is that an accurately weighed sample undergoes burning at high temperature (900-1000°C) inside the combustion chamber, which releases carbon dioxide, water and nitrogen. The nitrogen is converted to nitrogen gas and oxides in the combustion chamber. Detection of the gases is carried out by a GC separation followed by quantification using thermal conductivity detector. Aspartic acid is used as the standard for calibration. Using a conversion factor of 6.25 according to the Dumas method (Cunniff & Association of Official Analytical, 1995; Mariotti, Tome, & Mirand, 2008) the detected nitrogen levels can be calculated to project protein levels.

A schematic figure describing the processes in the EPA nitrogen analyser from sample preparation to interpretation of results using software Eager 300 is illustrated in Figure 5.1.

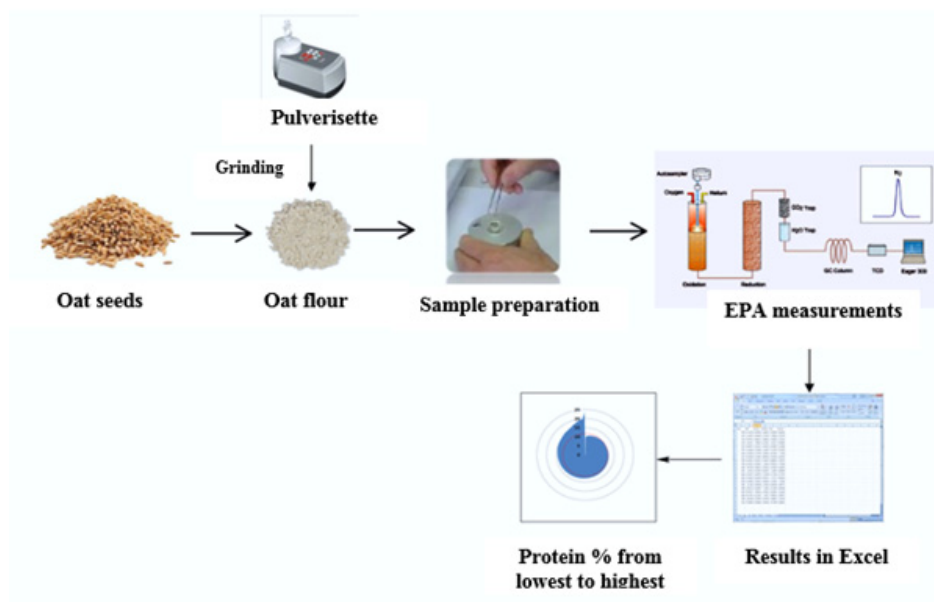


Figure 5.1

Schematic overview of the steps in protein quantification of TILLING oat lines. The seeds of TILLING oat lines were ground to a fine powder using the Pulverisette. The weighed samples in a tin capsule were placed in an auto-sampler in the analyser. Total protein percentages were calculated from the total nitrogen produced. Results were exported to an Excel sheet and protein percentages were compared with control. The high-protein oat lines were selected when the data was ranked from lowest to highest.

Amino acid analyser

A completely automated Biochrom analyser controlled by the Biochrom EZChrom Elite Data Processing Software was used in Papers II, III and V.

Amino acid analysis is a technique that measures the qualitative and quantitative compositional of individual amino acids present in a sample. The detection and quantitation of amino acids is a key part of protein and food analysis. The technique uses the ion exchange chromatographic method, the traditional cation flow chromatography procedure to separate underivatised amino acids (AAs), followed by the formation of coloured products with ninhydrin and their detection in the visible region. Beckman et al., received the Nobel Prize in Chemistry in 1972 for developing the fully automated amino acid analyser that was introduced in 1958 for their work on ribonuclease. The Biochrom amino acid analyser used for the present study followed the refined version of the traditional cation flow chromatographic procedures.

The amino acid samples are separated on the basis of pH and ionic strength of the various buffers pumped to the column. Inside the reaction coil, ninhydrin reagent at high temperature mixed with column eluent, amino acids, formed coloured products. The intensity of the colour produced, which is proportional to the quantity of amino acids, is detected by the photometer unit with wavelengths ranging from 570 nm to 440 nm.

The intensity of the signal from each amino acid is converted into a chromatographic peak. The area under the peak obtained for individual amino acid reflects the concentration of the amino acid, and retention time identifies each amino acid.

Asymmetrical flow field-flow fractionation (AF4)

Asymmetrical flow field-flow fractionation (AsFIFFF or AF4) was used to characterise protein complexes in high-protein oat lines (Paper III) as well as in heat treated and non-heat treated Kerstin oat variety (Paper V).

Field Flow Fractionation (FFF) is a flow-based separation methodology that was first introduced by Calvin Giddings in 1966 (Cao, Pollastrini, & Jiang, 2009; M.E. Schimpf, Caldwell, & Giddings, 2000; Martin E Schimpf & Giddings, 1987). The FFF contains an elongated flow chamber termed 'channel' (Figure 5.2a). There is no stationary phase, unlike other chromatography, and separation takes place in a liquid phase. The retention and separation of analytes occurs by the use of an external force field applied perpendicularly to the direction of sample flow through

(Dondi & Guiochon, 2012; Giddings, 1993; Liu, Li, & Giddings, 1993; M.E. Schimpf, Caldwell, & Giddings, 2000; Wahlund & Giddings, 1987). The laminar flow of sample inside the channel is governed by a parabolic flow in the FFF channel, and the flow rate of the analytes depends on the displacement velocity of the flow stream and the distance from the wall of the channel (channel width/flow depth, $B=H$). The flow velocity increases from near zero at the channel walls to a maximum at the centre of the channel (Figure 5.2b). When the perpendicularly applied force field drives the analytes towards the accumulation wall of the channel, a diffusive force builds up due to the concentration differences of the analytes and drives the analyte back towards the centre of the channel. When both the external force field and diffusion coefficient reach equilibrium states, a rapid analyte concentration profile is built up. When the flow velocity zones of the analyte are slower than the average velocity of the carrier liquid passing through the channel, retention and separation occur.

There are two types of flow FFF. (1) Symmetrical flow field-flow fractionation (FIFFF) and (2) asymmetrical flow field-flow fractionation (AsFIFFF or AF4). In this thesis, I used AF4. The AF4 instrument is very similar to that of the symmetrical technique. The only difference is that the cross flow in AF4 originates across the membrane frit assembly to prevent unwanted particles from entering provide a uniform flow, not by an external pump line as in FIFFF.

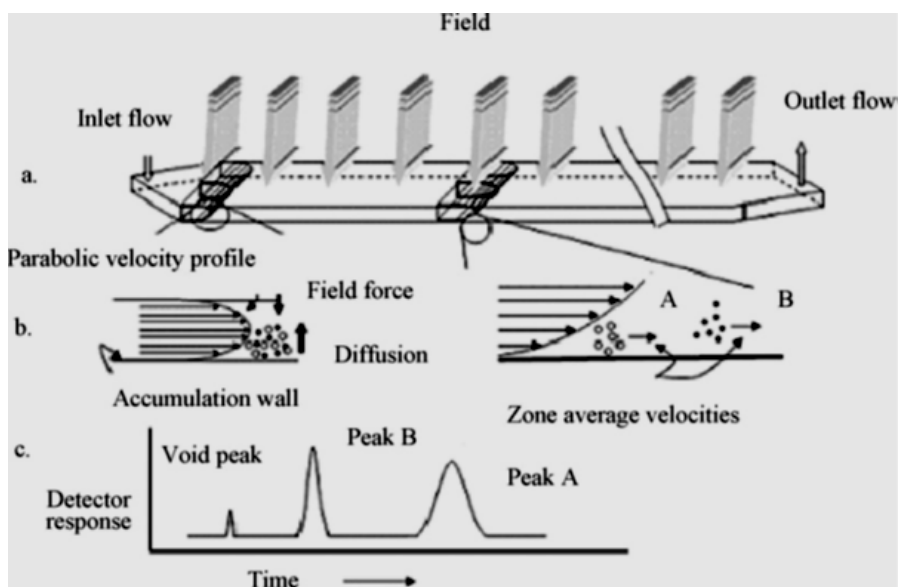


Figure 5.2

Schematic representation of FFF flow channel. (a) FFF channel cut out; (b). Detailed view of the normal mode of differential migration of two different particles A and B. The applied field (B) moves faster under higher elevation of faster flow velocity and elutes before A; (c) a typical FFF fractogram showing the characteristic elution order. The fractogram is a detector response versus elution time (or elution volume) curve.

Chapter 6. Results and discussion

Screening of oat TILLING population (Papers I-III)

One primary aim of this work was to identify high-protein lines from an oat TILLING (Targeting Induced Local Lesions in Genomes) population of the spring oat cultivar SW Belinda (Chawade, et al., 2010). Oat is an excellent source of high-quality protein with favourable amino acids. Normally, the protein content in oat is about 10%. Oat lines with a double protein content, approximately 20%, would have great potential as an alternative, vegetative protein source.

More than a thousand individual lines were screened for total protein, and assayed by an elemental particle analyser. This identified 230 lines with a seed protein content of 15% protein or higher. The highest line had 24%. The 15 highest of these lines were then selected for further studies. First, the protein content in individual seeds was determined. This confirmed the high protein levels and showed that, in some lines, the high-protein character segregated.

Crosses were performed between the six lines with the highest protein levels and the original non-mutated Belinda variety from which the mutagenised population was derived. The F1 hybrid seeds grown in the greenhouse and self-pollinated and individual seeds from the F2 offspring were analysed. This showed that the high-protein character was stably inherited. To further test the stability of high protein content, the 15 highest lines were amplified in the field and protein content was again determined in seeds harvested at the end of the season; the high protein character was confirmed. The mean (range) protein content of 15 high protein oat lines was 16.4% (15-19.1%) after field propagation.

The total protein was extracted and analysed by SDS-PAGE. This showed that the relative levels of individual proteins were different. A few lines clearly have elevated levels of globular proteins. Total dietary fibre (TDF) β -glucan and lipid levels were also measured in the selected lines. The samples contained mean (and range) 10.3% (8.3-14) total dietary fibre, 4.64% (3.2-5.42), β -glucan, 5.0% (3.3-6.5%) and lipid. This showed that the values for these components were normally distributed around the original level in Belinda and there was no significant negative correlation between fibre, β -glucan and lipid content and high protein content.

Discussion

We showed that mutation can increase the total protein concentration of oats. By screening 1050 oat lines, an increase in protein percent above 65% was identified in several lines compared to the original non-mutated SW Belinda cultivar. However, confirmation of stability of high protein content and its stable inheritance in the succeeding generation is very important. Consequently, an F2 population of high-protein lines was obtained after backcrossing with parental Belinda. Backcrossing helps to eliminate unwanted mutation to some extent.

The main components that make oat seeds superior to other cereals are dietary fibre and lipids, in combination with a high protein content. In order to investigate whether the high protein content in seeds has any impact on the availability of other proximate constituents, the total TDF, β -glucan and lipid content were also calculated. The concentration of other macromolecules was not affected by the high protein content, but individual protein classes did differ between lines, as shown by SDS-PAGE analysis. Bearing in mind that mutation is not categorised under GMO-regulation, development and utilisation of high-protein oat cultivar for food and feed use do not need any legal acceptance.

The identified high-protein oat lines were further characterised by amino acid analysis to determine the protein quality and concentration of the indispensable amino acids in the protein. In addition, physiochemical characterisation of soluble proteins using chromatographic methods were carried out. Also, change in solubility fractions between oat lines was evaluated. This has allowed us to compare solubility fractions between lines, which helped to identify the specific protein fraction elevated in high-protein oat lines due to mutation.

Conclusions: Identification of high-protein oat lines

- More than 230 lines with protein content $\geq 15\%$ were identified.
- Fifteen lines were selected in which the highest line had 24% protein.
- Crossing studies confirmed that high protein character segregates in the following generation.
- SDS-PAGE analysis showed that individual protein fractions differ between lines.
- Crossing and amplification of F1 hybrid seeds showed a stable inheritance of the high-protein character in the next generation.
- Total dietary fibre, β -glucan and lipid content were all similar to the control variety Belinda, and no correlation was found to high protein content.

Amino acid analysis of high-protein oat lines (Paper II, III)

To date, no oat varieties have been developed with sufficient levels of all nutritionally limiting amino acids like lysine, threonine, methionine and tryptophan, when considering the need of young children, which is the base for the FAO amino acid reference pattern used to quantify protein quality (FAO/WHO, 1991; Maruyama, Shands, Harper, & Sunde, 1975; D. M. Peterson, Brinegar, & Webster, 1986; Shewry, 2007; C. Zarkadas, 1982). The highest percentage of oat lysine reported so far is approximately 4.2%, which is higher than in other cereals, but still below the recommended FAO reference standard of 5.5% (FAO/WHO 1991). Methionine is another amino acid that is nutritionally limiting in oats and in other cereals. The highest level of methionine reported is 3.76% and 3.23% for Oxford and Sentinel respectively, two Canadian cultivars (C. Zarkadas, 1982).

Here the amino acid (AA) composition was determined for proteins isolated from 31 high-protein oat lines that ranged in seed flour protein level from approximately 18% to 24%, originally identified from the mutagenised population. The Belinda variety, from which the mutagenised population was derived, has approximately 12% protein and was used for comparison. In the selected high-protein lines, the total amino acid content ranged from 119.9 to 196.8 g/kg flour. The mean contents (g/kg protein) of each amino acid in these lines were lysine 37.30, threonine 34.14, phenylalanine 47.32, valine 45.61, histidine 21.36, glutamic acid 180.75, proline 49.23, glycine 46.23, alanine 43.03, cysteine 33.03, methionine 26.96, serine 40.63, tryptophan 10.33, arginine 61.45, aspartic acid 76.43, tyrosine 31.21, isoleucine 35.47 and leucine 69.61. However, the variability for individual amino acids was high, and several lines had both a higher content and a better balance of total essential amino acids than Belinda. The five amino acids (Lysine, Threonine, Leucine, Isoleucine and Valine) that may contribute to a low Glycaemic Index, (GI; Esteves de Oliveira, Pinheiro Volp, & Alfenas, 2011; Nilsson, Holst, & Bjorck, 2007) were higher in several high protein lines (CT2702, CT1424, CT1394, CT2700, CT1425 and CT1260). However, these lines were still limited in lysine content with the exception of one line, CT2702, where also the lysine level was high. When recalculated to g lysine per kg oat protein, the value obtained was 46.4, which means that the nutritional protein quality in this line reached the FAO recommended level for adults (Table 6.1).

Table 6.1

Recommended daily intake (RDI) of essential amino acids for humans (FAO 2007).

Age (y)	His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
0.5	20	32	66	57	27	52	31	8.5	43
1-2	28	31	63	52	25	46	27	7.0	41
3-14	16	30	61	48	23	41	25	6.6	40
15-18	16	30	60	47	23	40	24	6.3	40
>18	15	30	59	45	22	38	23	6	39
Range in oat lines	15.1-25.8	23.3-43.3	48.9-82.1	27.5-46.4	40.4-98.2	56.4-93.2	23.9-43.8	8.7-13.7	28.2-58.6

The values of the individual amino acids are given as nutritional quality of protein (g/kg total protein). The RDI varies at different ages.

The average nutritional quality for the high-protein oat lines (g/kg total oat protein) is shown at the bottom.

SAA, sulphur amino acid; AAA, aromatic amino acid, His, Histidine; Ile, Isoleucine; Leu, Leucine; Lys, Lysine; Thr, Threonine; Trp, Tryptophan; Val, Valine.

Discussion

The nutritional quality of dietary proteins is determined by the concentration of indispensable amino acids, i.e. histidine, isoleucine, leucine, lysine, methionine, phenyl alanine, threonine, tryptophan and valine. In this paper, we show that most of the lines have sufficient EAAs, in many cases higher than the FAO/WHO recommendation. The average maximum for lysine (the most limiting amino acid of oat) is 4.7% of the total protein. This is below the recommended 5.5% value (FAO/WHO, 1991). However, one line (CT 2702) had lysine at 97.36%, i.e. almost reaching the reference pattern for children over 3 years and adults. The increased protein content and improved amino acid composition of these nutritionally limiting amino acids and other amino acids with potential health effects indicated the usefulness of the mutation breeding procedure.

Conclusions: Amino acid content of high-protein oat lines

- Several of the high-protein lines contained relatively high concentrations of EAAs, e.g. lysine, threonine, and methionine. These are not only high in protein but also have a more favourable balance of amino acids.
- A comparison with the FAO/WHO recommendations of essential amino acids showed that although there was a slight variation between lines, only lysine was limited. However, in one high-protein line, lysine reached the recommended level for 3 year children or older.

Soluble proteins of high protein oat lines (Paper III)

In this paper we selected the best five high-protein oat lines that showed high protein and improved amino acid composition in the initial study (Paper I and II). Total and soluble protein percentages and relative percent of soluble protein to total protein were quantified using EPA and asymmetrical flow field-flow fractionation (AF4). The results showed that all five lines had significantly increased soluble protein content compared with the parental cultivar Belinda. The average soluble oat protein levels obtained from the EPA measurements ranged from 14% (CT1410) to 9% (CT2830) for the high-protein oat lines compared to 4.4 % for Belinda. The relative amount of soluble protein (soluble protein/total protein) in each seed line was also calculated, and was found to be almost twice of that in Belinda (37.6%), ranging from 67% (CT1394) to 52.2% (CT2688). The average soluble oat protein levels obtained from the AF4 measurements range from 12.4% (CT1410) to 7.6% (CT2830) for the high-protein oat lines, compared to 3.0% for Belinda. This result confirmed the obtained values by EPA, and both the combined AF4 and the EPA results show that the high-protein oat lines have relatively higher amounts of soluble protein than Belinda.

The distribution of different solubility fractions and their molecular weights, calculated from the AF4-MALS/UV data, showed that the soluble protein percent of five high-protein oat lines was not only significantly higher in general but also that they were higher in albumin, globulin and protein aggregates compared to Belinda. The highest soluble protein content was found in CT1410 (12.4%), which is twice as high as in Belinda. The highest globulin levels were found in CT1410, CT1394, CT1260 and CT2830. The variation in the protein profile revealed by SDS-PAGE analysis (Paper I) corresponded well with the AF4 results.

The soluble proteins of high-protein oat lines were analysed for amino acid composition. In the oat extract samples, two lines showed a significant increase in their relative amounts of most limiting amino acids such as lysine and threonine. Lysine averaged 5.7% in CT1410 and 5.6% in CT1394, similar to the FAO reference standard of 5.5%. These lines also showed a significant increase for threonine, second limiting amino acid, which averaged 7.2% in CT 1394 and 4.2% in CT1410, higher than the FAO reference standard of 4.0%. Two other lines also showed significant increase in threonine. They are CT2830 (5.2%) and CT 1260 (4.5%). Significant increases in basic amino acids (lysine, arginine, and histidine) and aspartic acid in CT1410, CT1394 and CT1260 clearly indicates that the high grain protein percent with increased amount of above amino acids are mostly accounted with increased amount of globulin fraction. Two strong and definite protein bands appeared in the SDS-PAGE, representing α and β polypeptides of globulin,

indicating that relative increase in total as well as soluble proteins of above oat extracts is due to increased globulin content.

Discussion

The differences in protein quality in oats depend upon the proportion of different solubility fractions, and any change of proportion as a result of genetic or environmental factors will influence amino acid levels and composition and thereby alter the nutritional quality. Since an increase in total protein levels in oats primarily leads to an increase in the globulin fraction, which in turn leads to a better amino acid composition, raising protein levels seems to be desirable. The selected five high-protein oat lines are very good candidates for breeding high-protein oat cultivar with an improved amino acid composition.

Conclusion: Soluble protein characterisation of high-protein oat lines

- The five selected high protein oat lines had higher soluble protein percentages as shown by EPA and AF4- MALS/UV.
- Increase in protein percentage is mainly due to an increase in the globulin protein fraction.
- The amino acid content of soluble protein extracts of two oat lines was sufficiently high in lysine (5.6% and 5.5%) to meet and even exceed the recommended amino acid pattern of proteins suitable for adults (4.7%).

Avenin proteins of mutated oat lines (Paper IV)

Oat prolamins (avenins) are the most heterogeneous proteins, and show both intra- and interspecific heterogeneity among different genotypes (diploid, tetraploid and hexaploid *Avena* species). The family of avenin proteins showed a high degree of polymorphism in size and charge that is higher than oat globulin and albumin (L. S. Robert, Nozzolillo, Cudjoe, & Altosaar, 1983). Protein polymorphisms give different electrophoretic patterns and are therefore good markers for a direct analysis of cultivars obtained from diverse geographical areas. It is not only helpful for breeders attempting to sample different germplasms but also to discriminate between species at the genetic level and for authenticity verification of different cultivars.

In this study, 25 SW Belinda varieties (in groups of five) grown or developed under diverse conditions (in the field, in the greenhouse, in different provinces of China, mutated oat lines and nitrogen-treated) were characterised using SDS-PAGE technique. Total proteins were quantified using an elemental particle analyser. The results show that the varieties grown in the field (in Sweden 2009-2013) or in the

greenhouse (2011-2015) did not increase in protein percentage compared with the parental variety (12%). However, the varieties grown in different provinces of China exhibited a slightly increased total protein percentage (14%). Mutated variety or those cultivars treated with N₂, had significantly higher protein levels, clearly showing the effect of treatment on protein levels. In the mutated lines, the protein content ranged from 17.3 to 20.2%. Similarly, varieties treated with N₂ fertilisers showed higher protein levels and the highest variation in protein content, ranging from 15.1 to 24%.

Avenin proteins from varieties (five groups) extracted using 25% 2-chloroethanol and the collected fractions were characterised by SDS-PAGE. The SDS-PAGE revealed three allelic blocks or groups of Avenin called α , β and γ that showed five to eight bands with molecular weight between 15 to 66 KDa (S. I. Kim, Charbonnier, & Mosse, 1978). A diagrammatic representation was obtained according to the International Rules of Genetic Nomenclature for genotypic characterisation using avenin proteins (Portyanko, Sharopova, & Sozinov, 1998). The analysis showed that only one group of cultivars, those obtained from the Swedish field, possessed three allelic blocks or groups containing at least eight bands. The cultivars from the greenhouse, mutated as well as cultivars grown under excess N₂, had four bands. The cultivar obtained from China had four bands, one of which was a minor band that may have been caused by differences in band mobility, so it is not considered for comparison. However, four additional loci (one dense and three mild bands) were noticed in group I, which represented the cultivars grown in Swedish fields.

Discussion

The question of the storage protein composition and its quantification is still problematic. Oat globulin and avenin showed little variation in the pattern of electrophoretic bands in SDS-PAGE gel, and gave identical banding pattern for cultivars. Since all 25 varieties used for the present study were developed from one single cultivar, the varieties do not warrant any differences in globulin and albumin pattern. However, the avenin protein groups have size and charge heterogeneity that has been analysed for inheritance studies (Gregova, Šliková, & Hozlár, 2015; S. I. Kim, Charbonnier, & Mosse, 1978; McDonald, 1980; Portyanko, Sharopova, & Sozinov, 1998). The cultivars grown in Swedish fields in different years and location showed differences in allelic blocks compared to other groups grown in the greenhouse in different years.

Conclusions: Avenin proteins of mutated oat lines

- Mutated oat lines showed the same banding pattern for avenin protein groups as in the parental cultivar SW Belinda.
- Avenin protein of mutated oat lines can be used as a tool to identify the pattern of inheritance of high protein content in the succeeding generation after crossing and as a tool for genotypic identifications from a mixed population of *Avena* species.

Effect of heat treatment on soluble oat proteins (Paper V)

Thermal treatment is the most important common processing technique used for denaturing proteins. Protein denaturation occurs when a change in the spatial arrangement of polypeptide chain is introduced, which destroys internal bonds and causes a higher degree of disorder. Denaturation leads to loss of enzymatic activity, loss of solubility in solvents in which the protein previously was soluble and change in molecular weight. It also leads to the formation of aggregates that can be both soluble and insoluble.

In this paper, the change in total as well as soluble protein content of heat treated and non-heat treated Kerstin oat was determined. The heat treated samples were provided by FrebacoKvarn (Linköping, Sweden). The Kerstin oat groats were heat treated by steaming at 102 °C for 50 min followed by drying at 110 °C/120 °C for 50 min. Change in the soluble protein fraction and the destruction of various amino acids of both heat treated and non-heat treated samples were analysed using AF4-MALS/UV, amino acid analyser and SDS-PAGE. Heat treatment resulted in ~ 5% reduction in total protein by weight relative to defatted oat flour. The amount of soluble protein was quantified by integration of the UV fractogram. The reduction in the amount of soluble proteins in the heat treated oat showed a decrease in intensity of the UV traces compared with non-heat treated oats. We found that the total protein values ranged from 74.6 ± 5.3 wt. % in non-heat treated oats to 35.7 ± 4.5 wt. % in heat treated oats. Using the AF4 fractogram, differences in decreases in the peak areas of different populations corresponded to a decrease in monomeric (albumin) proteins and an increase in hexameric (globulin) aggregates. The ratio of monomeric to hexamer and aggregate proteins was reduced from 1.82 to 1.48 as a result of heat treatment, so the results indicate that solubility of monomeric proteins is sensitive to heat treatment.

The relative amount of amino acid in heat treated and non-heat treated oat using hydrolysis methods shows a significant reduction in several amino acids such as

threonine, proline and methionine. A reduction in leucine, alanine, lysine and aspartic acid was also observed. In the SDS-PAGE, a reduction in protein bands for heat treated compared to non-heat treated samples confirmed that the heat treatment affects the solubility of different protein fractions, which is in agreement with total protein and AF4-MALS/UV analysis. Protein bands that appeared in the SDS-PAGE and AF4 data, and which were visible in the non-heated but not in the heated sample, corresponded to molecular weights in the ranges from 14.4-27 kg/mol, 27-7 kg/mol, and 37-60 kg/mol, respectively. It is the loss of these proteins that may result in the reduction of the UV signal in population 1 (monomeric albumin) in the AF4 fractogram and the slight decrease in molar mass before 8-minute elution time (Figure 4). Also, loss of a protein band in the SDS-PAGE corresponding to water soluble oat prolamins between α and β subunits was observed only for the non-heat treated sample. These results suggest that heat treatment of the oats affects the solubility of both the albumin and prolamins.

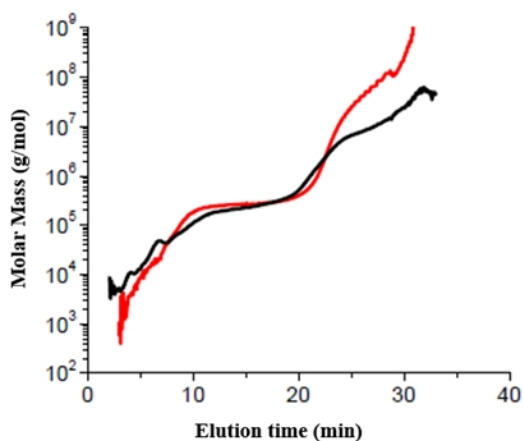


Figure 6.1

Molar mass distribution comparison between KHNT (black) and KHT (red) oat extracts. The increased M for the KHT sample compared to the K sample at elution times longer than 23 min occurs because of the decreased intensity of the UV signal resulting from reduced protein solubility in the heat treated sample.

Discussion

In this paper we show that heat treatment of total as well as soluble oat proteins will negatively affect both quality and quantity. The reduction in protein decreases amino acid concentrations, including some of the essential amino acids, which reduces the nutritional value of the product. Selective elimination of albumin and water soluble prolamins fractions due to heat treatment indicates that both fractions are less thermally stable. The presence of oat globulin fractions both in heat treated

and non-heat treated samples confirmed that oat globulins are more thermally stable than the other protein fractions. These effects should be considered when food proteins undergo heat treatment.

Conclusions: Effect of heat treatment on the soluble proteins of oats

- Heat treatment significantly affects the solubility of oat proteins.
- Albumin and prolamin proteins are most sensitive to heat treatment.
- Oat globulin is more thermally stable compared to other protein fractions.
- Reduction of several essential amino acids occurred during heat treatment. This leads to a change in functional properties of the proteins and a decrease in nutritional value of the final product.

Concluding remarks

There is growing evidence that an optimum intake of dietary proteins is necessary to sustain health. Amino acid composition of the protein source compared with the nutritional need is the crucial factor for effective utilisation of protein sources.

During the course of this work, more than one thousand mutated oat lines were screened to identify oat lines containing high-protein levels. A number of lines were identified with protein levels of 20% or higher. However, it is important to investigate whether the genetic event that caused the increase in protein content inversely affected other important macromolecules in the oat seed, such as dietary fibres, β -glucans and lipid content. It is also important to characterise the stability of the high-protein character in the field, the inheritance and the segregation pattern. Our results showed that there was no positive or negative correlation between fibre, beta-glucan, lipid content and high protein content. Also, the high-protein nature was stably inherited to the next generation. These findings are very important if we are to meet consumer needs for high-protein oat products that are still rich in β -glucan and fat content.

After the identification of the high protein content, the quality of protein was a major concern. Quality can mean different things, and many approaches have been used to characterise protein quality. The nutritional quality of dietary protein is determined by the concentrations of the indispensable amino acids in the protein. Here, total amino acid analysis as well as a characterisation of soluble proteins was carried out to determine protein quality in the selected high-protein lines. After the analysis it was clear that several of the high-protein oat lines presented here show a well-balanced amino acid profile with elevated lysine content, in some cases higher than the FAO/WHO recommendations. The five amino acids previously shown to contribute to a low Glycaemic Index (GI) were also higher in some of the high-protein lines.

The differences in protein quality reflect, to some extent, the proportion of various protein fractions in oat. When the protein content of oat varies as a result of agronomic or other factors, changes in their amino acid composition of soluble proteins may also occur. This in turn can affect the overall quality of protein because different solubility fractions have different proportion of essential amino acids. Using AsFIFFF (AF4) and SDS-PAGE, several of the high-protein oat lines with a high globulin content were rich in lysine, the most limiting amino acid of oat. These

findings of high-protein oat lines with high globulin content is very promising, as they could be an important resource for a further development of commercial oat varieties with enhanced protein nutritional qualities.

In Paper IV another important protein property was studied in the high-protein oat lines. The inheritance of avenin proteins was characterised using SDS-PAGE. It was shown that the particular mutations that gave rise to the high protein levels do not affect the avenin fractions in these lines. This is an important result because avenin can be used as a marker or as a tool to unravel the nature of inheritance of high protein content and stability in the succeeding generations after cross pollination. If 50% of the progeny could not inherit the avenin patterns of one of the parents after crossing, this indicates genotypic variation (S. Kim, Saur, & Mossé, 1979). It may also facilitate differentiation of our mutated oat population with other hexaploid oats when a mixture of *Avena* genus occurs.

While the higher thermal stability of oat globulin is favourable for some food use, heat treatment results in a 50% reduction in the availability of soluble protein, including several favourable amino acids. Using AF4 and amino acid analysis, it was found the albumin and water soluble prolamin proteins were less stable during heat treatment, resulting in loss of several of the important amino acids such as threonine, proline, methionine, leucine, alanine, lysine and aspartic acid.

In this thesis, I demonstrate the possibility of developing oat varieties with protein levels of 20% or higher. Several of the selected high-protein oat lines presented here showed a well-balanced amino acid profile with elevated lysine levels, and they still have high TDF, β -glucan and lipid content. Stability of high-protein oat lines and the stable inheritance of high protein content with an increase in the amount of good quality globular proteins were also confirmed. These lines will be an important resource in the work to meet consumer needs for high-protein oat products that are rich in β -glucan and fat content. Such varieties would be an important part of novel food products based on oats, and will also provide a serious vegetative protein alternative to pea, beans and soy bean.

Future perspectives

The main focus in this thesis was to identify high-protein oat lines from a mutagenised oat population.

- In future research it will be important to identify the specific mutations that underlie the high protein contents. This can be achieved through genotyping, by sequencing using high-throughput short-read sequences (ca 150 bp) and identifying SNP markers of high protein content or by quantifying differences in transcription of specific genes in lines with high and low levels of protein. However, since the oat genome is very large and complicated, it will be a great challenge to go from differentially expressed transcripts to a chromosomal location of a specific mutation. However, advanced bioinformatics may help us to define the molecular mechanisms underlying the property.
- It will be important to develop homozygous, stable high-protein oat varieties from the lines presented here. This will need several backcrossing and selection steps, thereby eliminating negative mutations in the succeeding generations.
- Avenanthramides and other phenolic derivatives, health-promoting minor components in selected high-protein oat lines, should be determined. This will help us to enhance beneficial health and other functional properties in the high-protein oat lines.
- It is also important to test the functional properties of high-protein lines such as emulsifying and gelling properties derived from higher protein content and altered protein quality in an industrial setting. This may lead to the development of new oat-based products with more proteins and better amino acid composition.
- An examination of individual protein fractions, especially globulin fractions from high protein oat lines, are of interest. This will help to identify the physical and functional characteristics, such as solubility, emulsifying properties, fat binding capacity, and water hydration capacity and their possible use as functional additives in different food systems. This will help expand the utilisation of the high protein oats.

- Nutritional studies with human subjects are very important to show beneficial effects of the increased amount of essential amino acids.
- The nitrogen absorbing capacity of mutated oat lines should also be quantified. Such a crop would be very useful in organic cultivation systems where nitrogen may be limited.
- Disease resistance, milling quality and groat percentages of selected lines should be tested.

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of her you ended up your attitude with a great laugh- apart from Karthikeya. Now Sreekar too???...Those memories are really love to remember!

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This picture shows the cross pollination in oats.
 It is taken from Landlantbruk (Country Farm), a
 Swedish Newspaper on Agriculture and Forestry.