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Fullnýting próteina úr grásleppu

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Líftækni og lífefni

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Report summary

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Titill / Title	**Fullnýting próteina úr grásleppu** / Full utilization of proteins from Lumpfish		
Höfundar / Authors	Margrét Geirsdóttir		
Skýrsla / Report no.	12-15	*Útgáfudagur / Date:*	Desember 2015
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Ágrip á íslensku:	Markmið verkefnisins var að þróa nýjar próteinafurðir úr hráefni sem fellur til við vinnslu grásleppuhrogna. Á þann hátt var stefnt að því að ná enn meiri verðmætum úr hráefninu með því að framleiða verðmætar próteinafurðir úr grásleppu. Í verkefninu var þróun þriggja afurða könnuð, 1) einangruð prótein fyrir surimi, 2) þurrkuð prótein sem íblöndunarefni og 3) vatnsrofin prótein sem íblöndunar og/eða fæðubótarefni. Illa gekk að einangra prótein úr grásleppuholdi en niðurstöður úr lífvirknimælingum á afurðum úr vatnsrofnum próteinum lofa góðu fyrir áframhaldandi rannsóknir.		
Lykilorð á íslensku:	*Grásleppa, aukið verðmæti sjávarfangs, nýjar afurðir, lífvirkni, prótein*		
Summary in English:	The aim of the project was to develop new products from lump fish to increase the yield and value of the catch. In the project the aim was to develop three types of products: 1) isolated proteins for surimi, 2) dry proteins as additives and 3) hydrolysed proteins as additives and/or food supplements. The project revealed that protein isolation from lump fish is difficult but hydrolysed proteins showed promising bioactive properties.		
English keywords:	*Lump fish, increased value, new products, bioactivity, proteins*		

Efnisyfirlit

1 Inngangur

Hefðbundin nýting á grásleppu hefur verið til hrognavinnslu en aðrir hlutar fisksins hafa ekki verið nýttir og varpað fyrir borð. Samkvæmt reglugerð sjávarútvegs- og landbúnaðarráðuneytis um hrognkelsaveiðar sem tók gildi 1. Janúar 2012 þá ber grásleppusjómönnum að koma með allan afla að landi (Sjávarútvegsráðuneyti, 2010). Því er afar mikilvægt að finna sem flestar nýtingarleiðir fyrir grásleppuna og einnig að hafa þær sem arðbærastar fyrir iðnaðinn.

Samkvæmt upplýsingum á heimasíðu AVS rannsóknasjóðs í sjávarútvegi (2012) hafa nokkur verkefni verið styrkt af sjóðnum er miða að fullnýtingu hrognkelsa. Rannsakaðir hafa verið eiginleikar grásleppuhvelju m.t.t. framleiðslu á kollageni og úr grásleppuholdi hafa verið framleiddir bragðkjarnar en einnig hefur með tilstyrk sjóðsins verið hafin sala á frystri grásleppuhvelju með holdi til Kína með góðum árangri. Einnig var verkefnið „Þróun vinnslu grásleppu“ unnið hjá Matís þar sem markmiðið var að þróa „hefðbundnar“ afurðir úr grásleppu (Gunnar Þórðarson o.fl., 2013).

Minni vinna hefur verið á sviði nýtingar á próteinum úr grásleppu, hvorki innanlands né erlendis, til að framleiða afurðir eins og t.d. surimi og fæðubótarefni sem mikill markaður er fyrir. Þessu verkefni var ætlað að bæta úr því. Því fleiri nýtingarmöguleikar sem finnast á grásleppunni því betra, en talið er að um 4000 tonn af fiski hafi verið hent árið 2010 (mbl 2010). Sem dæmi er nú eftirspurn eftir surimi í heiminum um 150 þúsund tonn umfram framboð, og hafa á síðustu árum margvíslegar óhefðbundnar tegundir verið nýttar í surimivinnslu til að mæta aukinni eftirspurn. Væri því eftir miklu að slægjast ef tækist að nýta grásleppupróteín í surimi. Þótt ákveðinn árangur hafi náðst við nýtingu á grásleppuhvelju með sölu til Kína er mikilvægt er að skapa fleiri nýtingarmöguleika til dæmis að þróa ferla til að vinna prótein úr grásleppu til nota í surimi og líkar afurðir, sem íblöndunarefni eða fæðubótarefni. Slík nýting á grásleppunni myndi þýða meiri verðmætasköpun en fæst í dag fyrir aflann. Þessu verkefni var ætlað að kanna þessa möguleika.

Samsetning grásleppu hefur verið mæld og helstu niðurstöður sýndu að meðal hrognafylling var 28%, haus, hvelja og sporður um 37% og flök um 13% (Tafla 1). Þegar hrogn eru nýtt er því um 70% af heildarþyngd fargað.

Tafla 1 – Niðurstöður mælinga á nýtingu grásleppu (Ólafur Reykdal o.fl. 2011). Meðaltal (n=10) ± staðalfrávik.

Veiðisvæði*	Veiðitími	Lengd [cm]	Heildar-þyngd [g]	Flök [g]	Hrogn [g]	Lifur [g]	Haus, hvelja og sporður [g]	Hryggur [g]	Slóg [g]
Húnaflói	20.3.2011	42±2	2887±584	396±65	774±247	99±36	1080±240	222±57	172±66
Húnaflói	25.4.2011	41±1	2842±390	412±73	716±185	109±36	1097±138	149±22	309±415
Húnaflói	7.6.2011	41±1	3148±279	454±71	940±118	97±14	1173±132	167±39	149±21
Skagafjörður	24.5.2011	39±2	2225±370	248±60	557±218	55±20	822±106	143±30	104±18
Skjálfandi	13.5.2011	39±2	2750±511	325±68	821±177	77±27	915±167	123±30	136±25
Hlutfall af heildarþyngd [%]				13,2	27,5	3,2	36,7	5,8	6,3

*sýni frá Húnaflóa unnin fersk en hin fryst

Efnainnhald grásleppa hefur verið mælt af Matís ohf. (Tafla 2) og reyndist prótein í flökum vera um 8,5% en 13,5% í hvelju (Ólafur Reykdal o.fl. 2011). Það er því ljóst að hægt er að ná að vinna umtalsvert magn af próteinum úr grásleppu til aukinnar verðmætasköpunar.

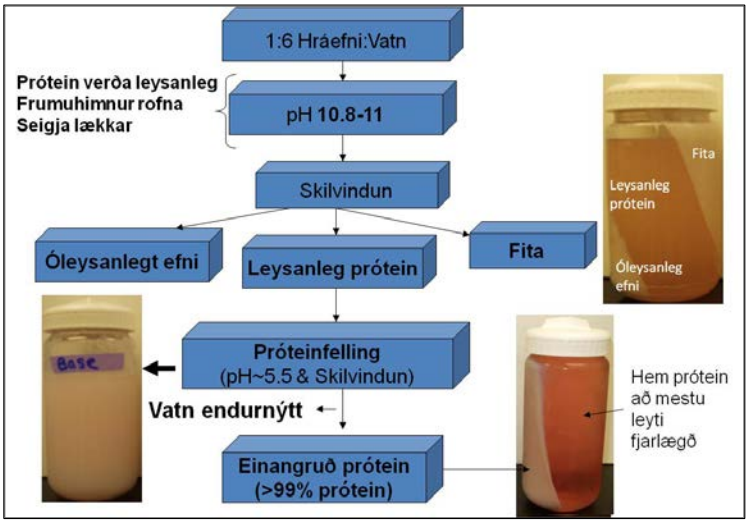
Tafla 2 - Efnasamsetning í grásleppuafurðum. Innihald í 100g af ætum hluta (Ólafur Reykdal o.fl. 2011)

Hlutur	Prótein [g/100 g]	Fita [g/100 g]	Aska [g/100 g]	Vatn [g/100 g]
Flök	8,5±0,6	13,3±3,5	0,9±0,1	77,6±4,2
Hvelja	13,5±3,2	0,8±0,6	1,0±0,1	82,6±3,4
Soðin hvelja	14,1	0,2	0,9	83,9
Lifur	9,8	22,1	1,3	66,3
Hrogn	13,5±1,6	3,4±0,7	1,0±0,1	80,5±2,0

Í verkefninu voru tvær mismunandi aðferðir notaðar við að þróa nýjar afurðir úr grásleppu. Annars vegar framleiðsla á einangruðum próteinum m.a. til að framleiða surimi og hins vegar framleiðsla á þurrkuðum próteinum með eða án forvinnslu með ensímum til að nota sem íblöndunarefni eða í fæðubótarefni.

Vöruþróun í verkefninu sækir hugmyndir í framleiðslu á afurðum úr surimi og nýtir þekkta en nýstárlega aðferð við einangrun próteina eða pH hliðrunaraðferð („pH shift process“). Mikil þekking er erlendis á sviði framleiðslu surimiafurða og pH shift ferli. Á Íslandi hefur sömuleiðis mikil þekking byggst upp á þessu sviði, aðallega innan Matís sem hefur unnið verkefni á þessu sviði í náinni samvinnu við þá erlendu aðila sem upphaflega þróuðu aðferðina og MPF Ísland sem hefur rétt á notkun aðferðarinnar á Íslandi. Í þessu verkefni var stefnt að því að sameina og nýta þessa þekkingu sem aflað hefur verið til að þróa nýjar verðmætar afurðir úr grásleppu.

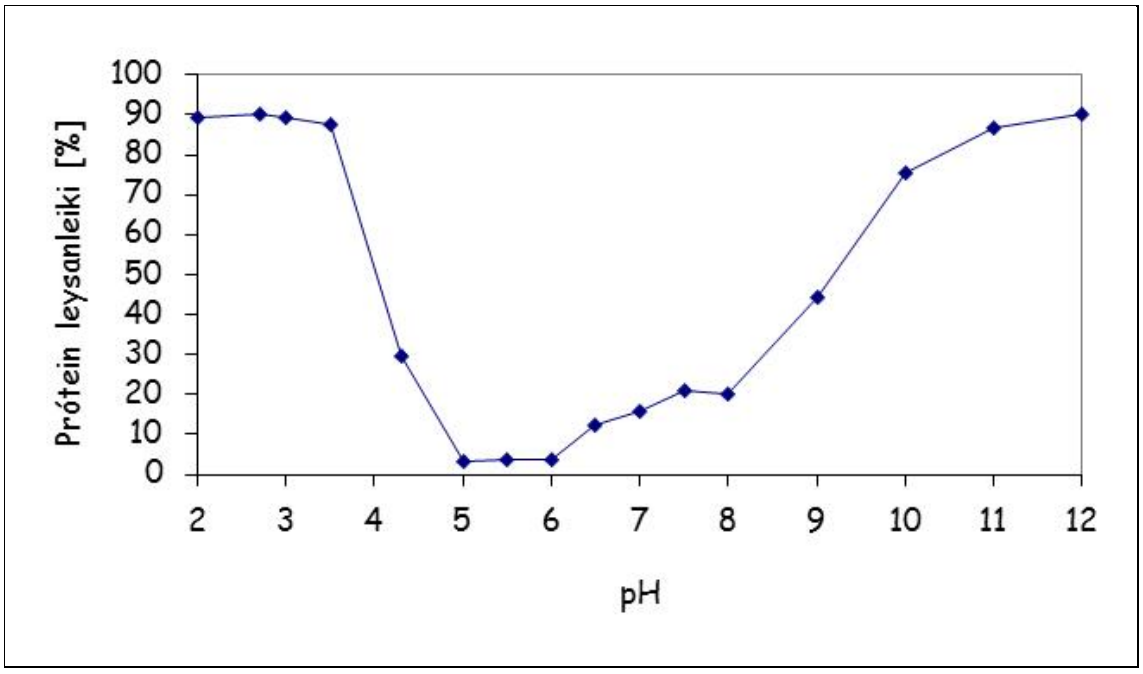
Próteineinangrunarferlið má sjá á Mynd 1. Ferlið gengur út á að þynna hráefnið með vatni, hækka sýrustigið til að gera eftirsótt prótein leysanleg, fjarlægja óæskileg óleysanleg efni (t.d. bein, roð og fitu) með skilvindun og fella að því loknu út hin æskilegu prótein með því að lækka sýrustigið (Hultin og félagar 2003). Forsendan fyrir gott surimi (Mynd 2) og prótein til íblöndunar er góð vatnsbinding og geljun próteinanna. Hægt er að sjá af efnainnihaldi grásleppu að fiskurinn inniheldur meira vatn per próteineiningu en flestar aðrar fisktegundir. Þetta er vísbending um að próteinsamsetning fisksins sé mjög sérstök og hafi mikla vatnsbindingareiginleika sem lofar góðu um vinnslueiginleika próteinanna og notkun þeirra í surimi og önnur matvæli eins og t.d. unnið fiskmeti.



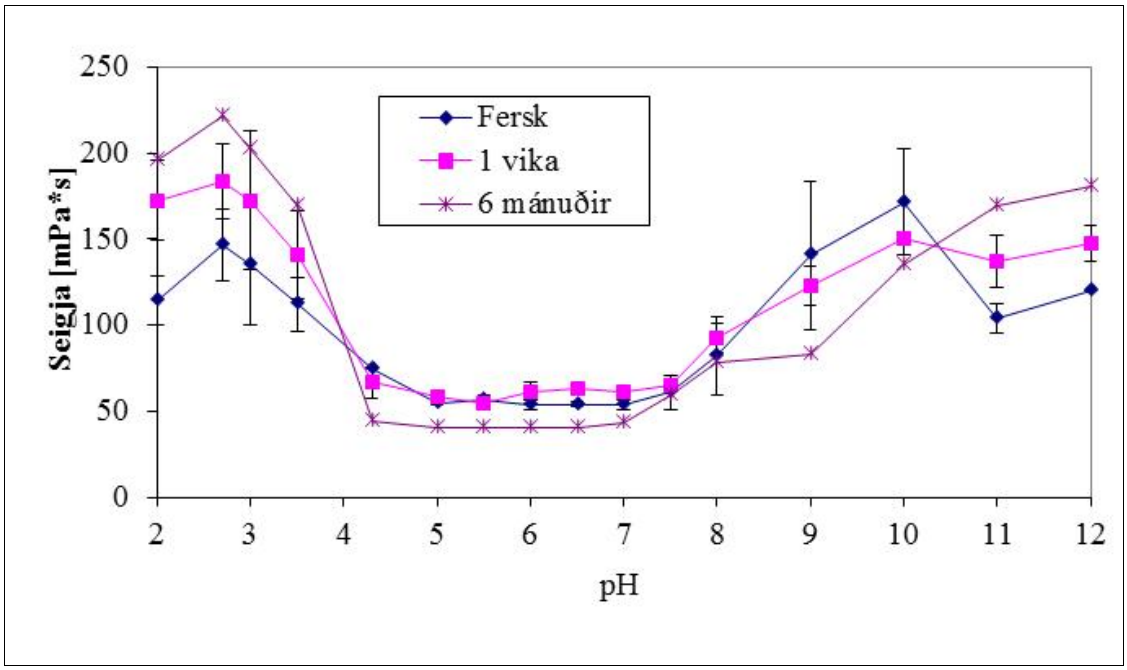
Mynd 1 - Próteineinangrunarferli með pH hliðrunaraðferð á tilraunastofu.

Mynd 2 - Dæmi um surimiafurðir

Sýrustigsaðferðin byggir á því að leysanleiki próteina er mismunandi eftir sýrustigi. Hefðbundinn ferill fyrir leysanleika fiskpróteina má sjá á Mynd 3. Þar sést hvar leysanleikinn er minnstur við um pH 5 sem kallast jafnhleðslupunktur próteinanna en mestur við hátt og lágt sýrustig. Við hvaða sýrustig þessi há- og lágmörk eru er misjafnt eftir því hvaða hráefni unnið er með. Einnig skiptir máli meðhöndlun hráefnis fyrir vinnsluna og er þar sérstaklegt horft til seigju lausnarinnar, en þar getur frysting haft áhrif á eiginleika próteina (Mynd 4).



Mynd 3 – Próteinleysanleiki síldarvöðva milli pH 2 og 12, við 4-6°C (Geirsdóttir o.fl., 2007).



Mynd 4 – Seigja síldarpróteina úr ferskri síld, eftir viku frystigeymslu og 6 mánaða frystigeymslu við 4-6°C milli pH 2 og 12 (Geirsdóttir o.fl., 2007).

Á undanförnum árum hefur verið lögð mikil vinna í að auka nýtingu og gæði í íslenskum fiskiðnaði. Erfitt verður að auka arðsemi hinnar hefðbundnu vinnslu svo miklu nemi, komið er að ákveðnu þaki. Aðaláherslan og sóknarfærin er á sviði nýtingar á hráefni sem í dag fer í ódýrar afurðir og yfir í mun verðmætari afurðir. Enn fellur mjög mikið af vannýttu próteinríku hráefni til við fiskvinnslu á Íslandi, en hér eru mikil verðmæti að fara fyrir bí. Uppbygging á sviði aukinnar nýtingar og verðmætasköpunar á fiskipróteinum er því gríðarlega mikilvæg til að eiga möguleika á því að auka verðmæti íslensks sjávarfangs á næstu árum.

2 Einangrun á próteinum

Í þessu kafla er fjallað um niðurstöður sem tengjast aðallega fyrsta verkþætti verkefnisins - Hámörkun á breytum við einangrun með pH aðferð – en einnig var hráefni unnið fyrir næstu verkþætti. Stuðst var við aðferð Hultin og félaga (2003) við að einangra prótein úr grásleppu eins og kom fram í inngangi. Á þann hátt var vonast til að fjarlægja óæskilega þætti eins og fitu og fá hreinan próteinmassa sem hefði jákvæða eiginleika en samsetning grásleppu bendir til að próteinin hafi einstaka vatnsbindieiginleika.

2.1 Efni & aðferðir

2.1.1 Hráefni

Notuð var bæði fersk eða frosin grásleppa. Frosin blokkfryst heil grásleppa eða fersk slægð grásleppa kom á ís frá Drangsnesi.

2.1.2 Leysanleiki

Grásleppa afþýdd, hökkuð niður, 6 rúmmálum af ís köldu kranavatni bætt við hakkið (1:6, fiskhakk:vatn) og lausnin gerð einsleit með hakkara (Dynamic MF 2000). Lausn skipt upp í skilvinduglös og sýrustig stillt. Sett í skilvindu á 10.000xg í 20 mínútur og leysanlegt prótein mælt með Biuret aðferð (*Sjá nánar í viðauka*). Leysanleiki metinn sem prósent af heildarmagni próteins í upphaflegri lausn skv.

$$Leysanleiki\ [\%] = \frac{Leysanleg\ prótein}{Heildarmagn\ próteina} * 100$$

2.1.3 Prótein einangrun

Grásleppa afþýdd (ef geymd í frysti), haus, hvelja og sporður fjarlægður, hökkuð niður (með VCB-62), 6 rúmmálum af ísköldu kranavatni bætt við hakkið (1:6, fiskhakk:vatn) og lausnin gerð einsleit með hakkara (Dynamic MF 2000). Sýrustig stillt á pH 11,0 með 2M NaOH lausn. Lausn síuð og sýrustig lækkað niður í um 5,3 með 2M HCl til að fella út prótein og lausnin síuð í annað sinn. Einangruð prótein vigtuð, pakkað í poka sem var lofttæmdur og geymd í frysti (-24°C) (*Sjá nánar í viðauka).*

2.1.4 Rafdráttur

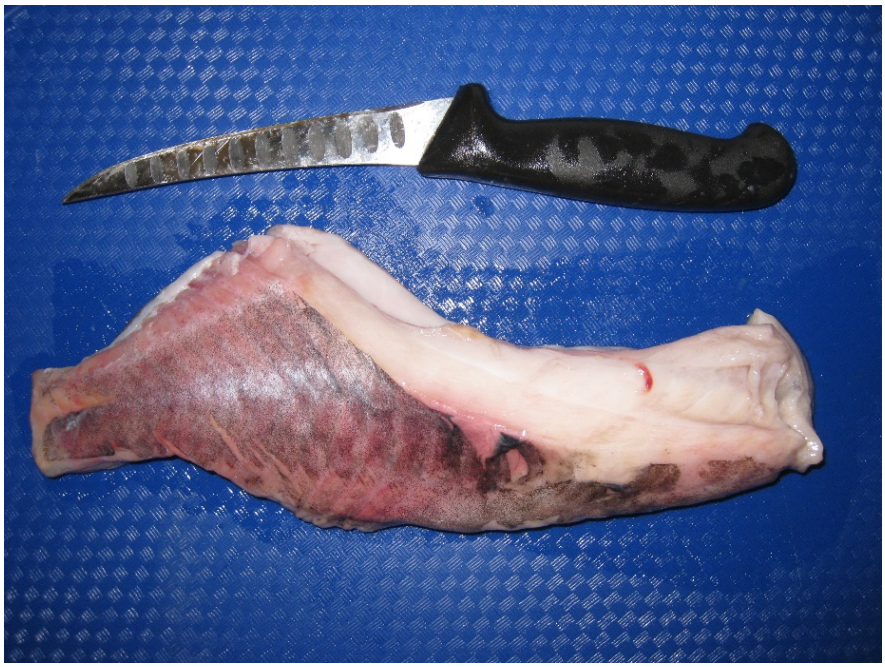
Samsetning einangraðra grásleppupróteina var skoðuð með rafdrætti (e. with sodium dodecyl sulfate polyacrylamide gel electrophoresis), oft kallað SDS-PAGE (Laemmli, 1970) og var framkvæmt með Mini-Protean Tetra system (BioRad). Valin sýni úr leysanleikakúrvu ásamt sýnum úr kalsíum útfellingartilraun voru útbúin til SDS-PAGE mælinga með því að blanda við Lamelli buffer og β-mercaptoethanol, soðið í 5 mín og svo kælt á ís. Sýni (styrkur u.þ.b. 1 mg/mL) og staðall (Neolab – cat. P7702S) voru sett á 12% akrílamíð gel og rafdregin við 60mA. Gelið var litað með Coomassie Blue, aflitað með 10% ediksýru og að lokum skannað til frekari úrvinnslu.

2.2 Niðurstöður og umræður

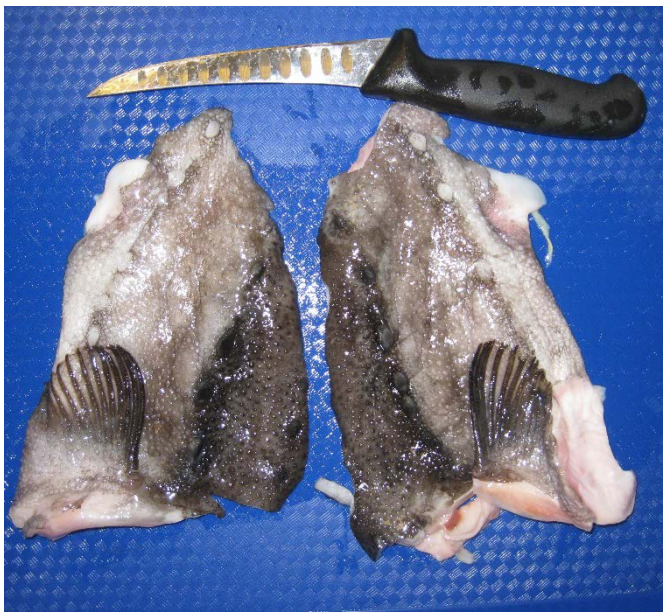
2.2.1 Forvinnsla

Bæði fersk og frosin grásleppa var notuð sem hráefni. Betur gekk að forvinna hráefni sem hafði verið fryst þar sem betur gekk að fjarlægja hveljuna. Mynd 5 - Mynd 9 sýna dæmi um grásleppur á mismunandi stigum forvinnslu. Sérstaklega var gaman að sjá hvað litur getur verið mismunandi á grásleppuhrognum (Mynd 9).

Mynd 5 – Grásleppa að lokinni slægingu.

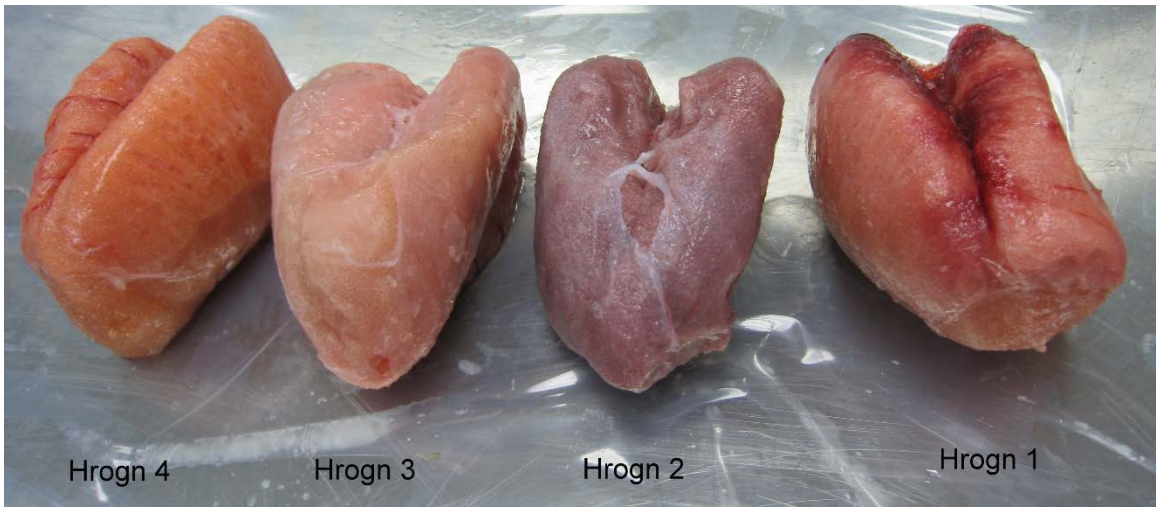


Mynd 6 – Grásleppuhold.



Mynd 7 – Haus og hvelja.

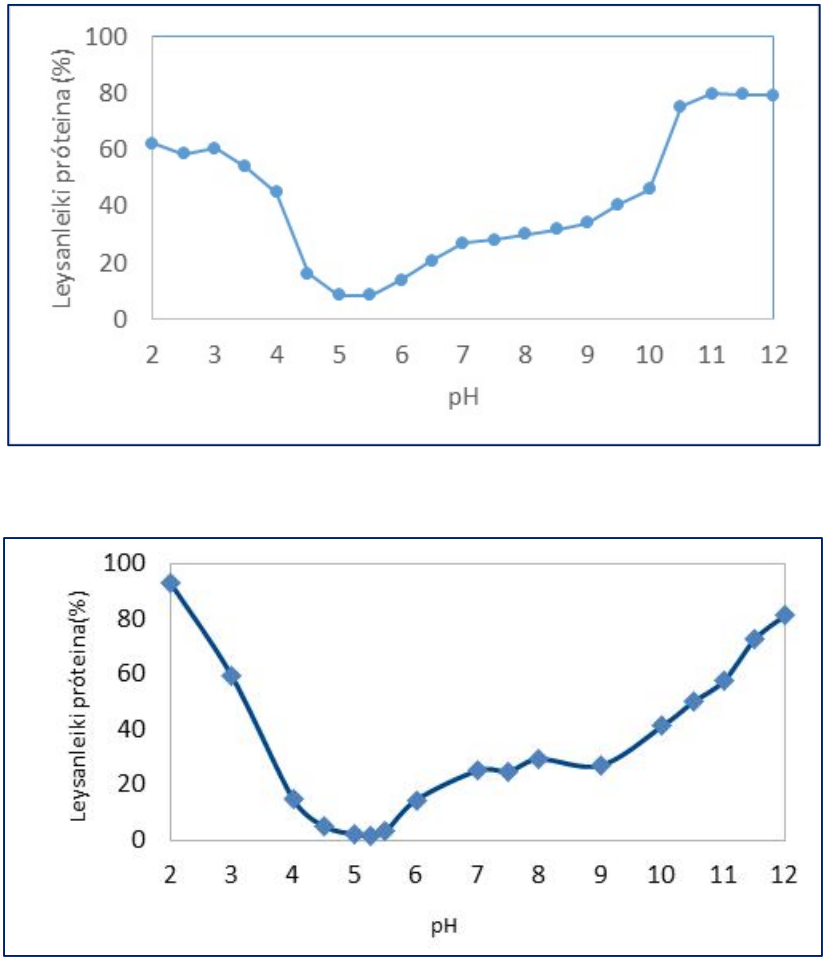
Mynd 8 – Grásleppur að lokinni hausun og slægingu.



Mynd 9 – Grásleppuhrogn.

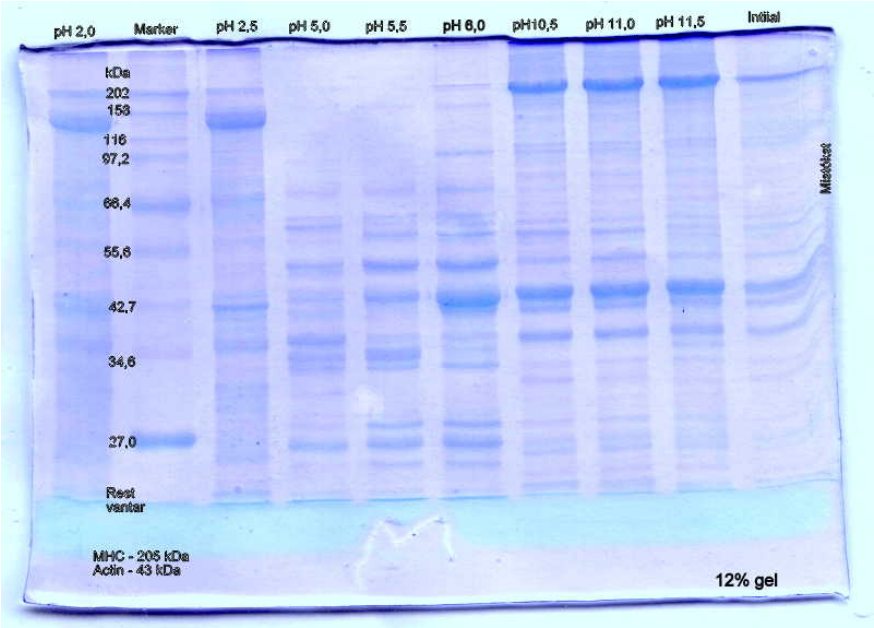
2.2.2 Próteinleysanleiki

Upphafssýrustig reyndist vera pH 7,5 sem er hærra gildi heldur en fengist hefur fyrir annan hvítan fisk svo sem þorsk og Tilapiu þar sem gildið er yfirleitt um 6,5 til 6,8. Þónokkur munur er á milli leysanlegra próteina í fersku og frystu hráefni (Mynd 10). Í fyrsta lagi er munur á leysanleika við pH 5,5 sem er meiri í fersku hráefni. Hins vegar var leysanleiki mun meiri við pH 2 fyrir frysta hráefnið. Prótein í fersku hráefni voru mun leysanlegri við pH 11 heldur en í frystu hráefni en það sýrustig er notað við einangrun. Sambærilegar niðurstöður fyrir leysanleika fyrir og eftir frystingu hafa áður sést þegar unnið er með annað hráefni til dæmis síld (Geirsdóttir o.fl. 2007).



Mynd 10 – Leysanleiki próteina úr ferskri (eftir mynd) og frystri (neðri mynd) grásleppu við valin pH gildi milli 2 og 12.

Prótein leysanleg við mismunandi sýrustig voru rafdregin sbr. Mynd 11.



Mynd 11 – SDS af leysanlegum grásleppupróteinum við nokkur sýrustig.

2.2.3 Próteineinangrun

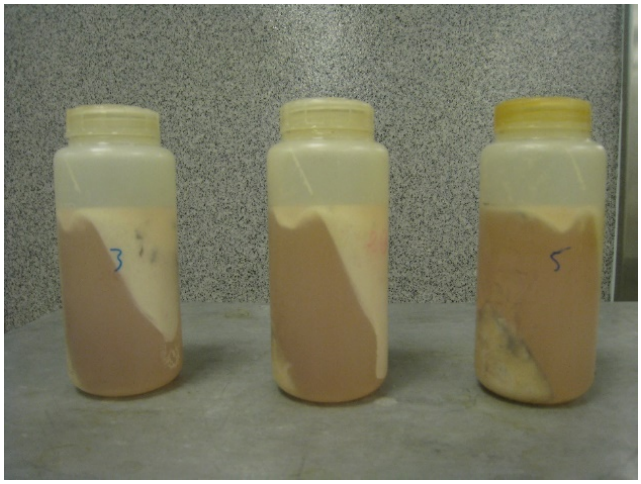
Fryst hráefni

Í fyrstu tilraunum var notast við fryst hráefni og féllu próteinin lítið út við pH 5,5. Það sem féll út var frekar eins og vatnsmikið mauk í stað þess að vera próteinmassi og var því ekki hæft í surimigerð. Nokkrar tilraunir voru gerðar en skiluðu fremur lágum heimtum eða um 30%. Var því ljóst að ekki væri unnt að nýta það til próteinframleiðslu. Próteinmassi sem var einangraður var því nýttur í ensímvatnsrof (sjá kafla 4.2 bls. 31).

Ferskt hráefni

Betri heimtur fengust þegar unnið var með ferskt hráefni en þó lægri en í fyrri tilraunir með annað hráefni eins og þorsk. Hinsvegar gekk ágætlega að fjarlægja rauðan lit úr blóðvökva (Mynd 12 til Mynd 14). Einnig var erfitt að einangra próteinin þar sem þau mynduðu ekki stórar þyrpingar við útfellingu heldur smáar agnir.

Mynd 12 – Hakkað hold og vatn í hlutföllum 1:6.

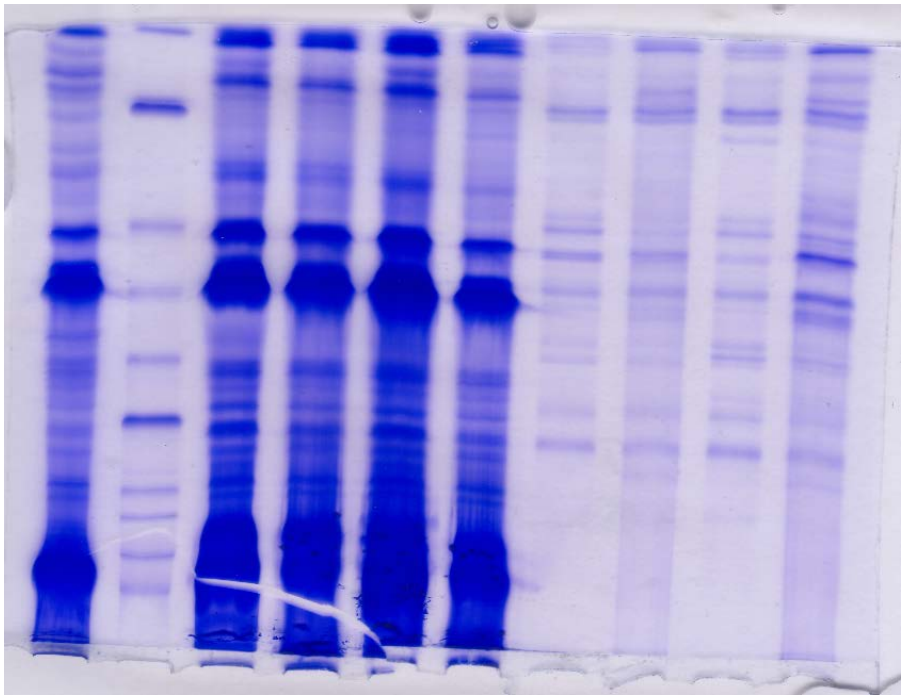


Mynd 13 – Hakkað hold og vatn í hlutföllum 1:6 að lokinni skilvindu við pH 11.

Mynd 14 – Lausn við pH 5,5 að lokinni skilvindu.

Ítarlegar tilraunir voru gerðar til að einangra prótein úr grásleppu með basaferli þar sem notast var við nýtt, ferskt hráefni. Vanalega falla prótein út við pH 5,5 en grásleppuprótein vildu ekki falla út í miklu magni. Er það óvenjulegt og hefur ekki sést fyrir aðrar fisktegundir sem hafa verið prófaðar eins og áður hefur komið fram. Reynt var að nota kalsíum í lausnina í styrknum 0,5% $CaCl_2$ en vitað er að það getur aukið útfellingu á próteinum. Framkvæmdur var rafdráttur (Mynd 15) á grásleppupróteinum til að kanna hvort önnur prótein væru þar heldur en í öðru fiskholdi. Enginn munur fannst á SDS mynd. Einnig leiddi hún í ljós að prótein féllu síður út með íblöndun á kalsíum. Á myndinni má sjá próteinrafdrátt á hökkuðum grásleppuflökum (hráefni), prótein sem féllu þó út við pH 5,0 og 5,5 með og án kalsíum og uppleyst prótein sem falla ekki út heldur haldast fjótandi í vatnslausn (supernatant). Niðurstaðan var að próteinin falla síður út með kalsíum þar sem línur 10 og 8 eru dekkri en línur 7 og 9 (Mynd 15). Einnig var próteinlausn látin vera yfir

nótt í kæli. Betri heimtur fengust á þennan hátt en ekki í það miklu magni að það teldist fýsilegt á framleiðsluskala.



Mynd 15 – SDS rafdráttur – línur frá vinstri til hægri: 1) upphafshráefni - grásleppa, 2) staðall, 3) grásleppupróteín við pH 5,0, 4) grásleppupróteín pH 5,0 með kalsíum, 5) grásleppupróteín pH 5,5, 6) grásleppupróteín pH 5,5 með kalsíum, 7) fljótandi við pH 5,0, 8) fljótandi við pH 5,0 með kalsíum, 9) fljótandi við pH 5,5 10) fljótandi við pH 5,5 með kalsíum.

2.3 Ályktun

Leysanleiki grásleppupróteina við mismunandi sýrustig reyndist vera svipað og fyrir önnur fiskprótein sem áður hafa verið prófuð. Hins vegar gekk illa að fella prótein út til að nýta í surimi eða aðrar próteinafurðir sem stefnt var á að þróa í verkefninu þrátt fyrir ítrekaðar tilraunir. Vatnsbindihæfni próteinanna eða fituinnihald í lausn eru aðallega talin vera ástæður og ekki tókst í verkefninu að finna lausnir á því. Af þessum sökum var ekki hægt að vinna að öðrum verkþætti verkefnisins – Þróun surimi úr grásleppupróteinum.

3 Eiginleikar þurrkaðrar grásleppu

Í þessum kafla er fjallað um niðurstöður sem tengjast Verkþáttum 3 & 4; Þróun þurrkaðra próteinafurða og Eiginleikar þurrkaðra afurða. Hér verður fjallað um vinnslueiginleika próteina þurrkuð beint. Í kafla 4 verður fjallað um eiginleika sýna að loknu vatnsrofi.

3.1 Framkvæmd

3.1.1 Grásleppuvinnsla

Heil frosin grásleppa var fengin frá Patreksfirði (Snerpa) í lok maí 2013 (5-6 kassar). Frauðkössunum var komið fyrir í -24°C og þeir geymdir þar til notkun fór fram. Grásleppan var tekin úr frysti 9.des.2013 og sett í kæli við 0-2 °C. Daginn eftir var hún tekin úr kæli og komið fyrir inn í vinnslusal við stofuhita (13 fiskar). Fiskurinn var hausaður, slægður og hvelja tekin af. Mismunandi hlutar voru hakkaðir og hveljan skorin í bita. Allt sett í bakka, fryst og frostþurrkað.

3.1.2 Efnamælingar

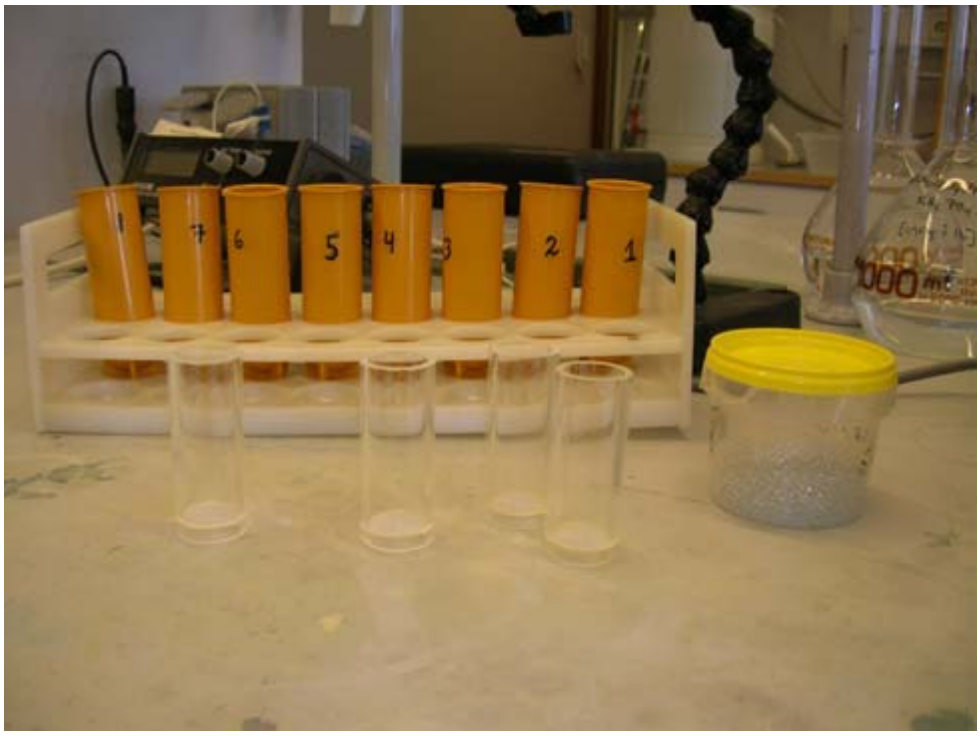
Vatn, prótein, fita, salt og aska mæld á efnastofu Matís í hráefni og þurrkuðum afurðum. Sýni voru send í amínósýrugreiningu til Þýskalands.

3.1.3 Vinnslueiginleikar

Vatnsheldni (Water holding capacity)

Um það bil 3,2 grömm af dufti blandað við 100 g af þorskhakki og 20 g af eimuðu vatni í matvinnsluvél í 30 sekúndur á hraða 5. Sýni látið bíða á ís í 30 mínútur. Vatnsheldni var síðan ákvörðuð samkvæmt skilvinduaðferð (centrifugation method) (Eide og fleiri 1982). Sýni (2 g) var nákvæmlega vegið í glæran hólk með neti í botni, sett í skilvinduglös með glerkúlum (Mynd 16) og skilvindað tafarlaust við 210 x g í 5 mínútur við 0-5°C. Þyngdartap eftir skilvindun var deilt með vatnsinnihaldi þorskhakksins og tjáð sem %WHC. Hvert sýni var mælt fjórum sinnum. Vatnsheldi reiknuð skv.

$$WHC\ [\%] = \frac{\text{þyngd sýnis} * vatnsinnihald\ [\%] - \text{þyngdartap}}{\text{þyngdartap} * vatnsinnihald\ [\%]} \cdot 100$$



Mynd 16 – Sýnaglös o.fl. fyrir vatnsheldnimælingar.

Olíubinding (oil binding capacity)

Um 5 g af dufti vigtuð nákvæmlega og 20 g olía sett í skilvinduglas (Beuchat, 1977). Lausnin látin standa við herbergishita í 30 mín, blandað með spatúlu á 10 mín fresti. Skilvindað við 4000 rpm í 30 mín, við hitastig um 20°C. Olíufasanum hellt af og hann veginn nákvæmlega. Olíubinding (OBC) reiknuð samkvæmt

$$OBC = \frac{heildarmagn\ olíu\ [g] - magn\ af\ olíu\ hellt\ af\ [g]}{massi\ prótein\ [g]}$$

Ýruhæfni (emulsion capacity)

Um eitt gramm af dufti vegið nákvæmlega og 100 mL af 0,1 M NaCl voru sett í 1L plastbikarglas (Kristinsson og Rasco, 2000; Webb og fleiri, 1970). Elektróðum fjölmælis, sem mældi viðnám (Ω), var komið fyrir innan á plastbikarglasinu. Sýnið blandað með UltraTurrax á 9500rpm í 20 sekúndur, án þess að snerta botn plastbikarglassins. Hraði

Ultra Turrax aukinn í 13500rpm, án þess að snerta botn plastbikarglassins. Wesson Vegetable Oil látin renna úr 500 mL skiltrekt, ofan í plastbikarglasið, til að skapa olíu í vatni ýrulausn. Viðnámið hækkaði skyndilega, ýrulausnin féll og olíurennslið var stöðvað. Á þessum punkti, þegar viðnámið hækkaði og ýrulausnin féll, hafði ýruhæfni próteinanna náð hámarki og myndað vatn í olíu ýrulausn. Hvert sýni mælt tvisvar sinnum. Ýruhæfni (EC) reiknuð samkvæmt:

$$EC = \frac{massi\ eftir\ blöndun\ [g] - massi\ fyrir\ blöndun\ [g]}{massi\ prótein\ [g]} / eðlismassi\ olíu\ [\frac{g}{mL}]$$

Ýrustöðugleiki (emulsion stability)

Um eitt gramm af dufti vegið nákvæmlega, 100 mL af 0,1 M NaCl og 100 mL af Wesson Vegetable Oil voru sett í 1 L plastbikarglas (Kristinsson og Rasco, 2000; Miller og Groninger, 1976; Yasumatsu og félagar, 1972). Sýnið blandað með UltraTurrax á 13500rpm í 2 mínútur. Sýninu hellt í 3 mæliglös (50 mL) og látið standa í 15 mínútur. Heildarrúmmál og rúmmál vatnsfasa lesið af kvarða mæliglassins. Hvert sýni var mælt þrisvar sinnum. Ýrustöðugleiki (ES) reiknaður samkvæmt:

$$ES = \frac{(heildarrúmmál\ [mL] - vatnsfasi\ [ml]) * 100}{heildarrúmmál\ [mL]}$$

Leysanleiki

Leysanleiki próteinduftsins í vatni, var metinn með Kjeldahl aðferð. Um það bil 2 g af dufti vegin nákvæmlega, leyst upp í 190 mL af eimuðu vatni og blandað með UltraTurrax á 8000rpm í 30 sekúndur. Sýnið látið bíða á ís í 60 mínútur. Skilvindað við 16300 x g í 15 mínútur @ 4°C í Bechman Coulter Avanti J-20 XPI skilvindu með JA – 10 rótor. Magn af leysanlegu próteini í vökvafasanum var ákvarðað með aðferð Kjeldahl (Nx6,25). Próteinleysanleiki reiknaður skv.

$$Próteinleysanleiki\ [\%] = \frac{Prótein\ í\ lausn\ [g]}{Heildarmagn\ próteina\ [g]} \cdot 100$$

3.2 Niðurstöður rannsóknar á þurrkuðum grásleppuhlutum

3.2.1 Grásleppuvinnsla

Heill fiskur var vigtaður eftir slægingu áður en haus var sagaður af með bandsög Matís og vigtaður (Tafla 3). Haus reyndist vera tæp 20% af heildarvigt fisksins. Að lokum voru hausarnir hakkaðir, settir í bakka, frystir og frostþurrkaðir (Tafla 3 & Mynd 17). Hrogn og innyfli voru einnig fjarlægð (Mynd 9). Hveljan var fjarlægð (Mynd 18) og reynt að hakka hana en án árangurs – reyndir voru 2 mismunandi hakkarar sem réðu ekki við hveljuna. Var hún því skorin í bita og frostþurrkuð þannig. Að lokum var holdið sem eftir var hakkað, fryst og frostþurrkað. Var ákveðið að hakka holdið með beingarði (Mynd 6).

Tafla 3 – þyngd af slægðri grásleppu og hausum.

Fiskur nr.	Heill fiskur [g]	Haus [g]	Haus sem hlutfall af heildarþyngd [%]
1	2366	473	20%
2	2618	377	14%
3	3139	350	11%
4	2673	463	17%
5	2178	416	19%
6	2642	445	17%
7	2770	493	18%
8	3177	548	17%
9	2691	485	18%
10	2426	466	19%
11	2797	444	16%
12	3356	522	16%
13	2695	436	16%
Meðaltal	**2733**	**455**	**17%**
Staðalfrávik	**332**	**54**	**2%**


Mynd 17 – Grásleppuhausar, fyrir og eftir hökkun.


Mynd 18 – Hold og hvelja að lokinni hausun og slægingu.

3.2.2 Þurrkun

Sýni voru vigtuð fyrir og eftir frostþurrkun (Tafla 4, Tafla 5 og Tafla 6). Hrogn og innyfli voru einnig skilin frá. Hrognin voru svo mismunandi á litin að ákveðið var að taka frá 4 mismunandi „liti“ og skoða nánar. Hrognin voru merkt 1, 2, 3 og 4 (Mynd 9) hökkuð, fryst og frostþurrkuð. Hveljan var eins og frauðplast að lokinni hökkun og ekki var hægt að hakka hana eða greina frekar.

Tafla 4 – Hakkaðir hausar, vigtun fyrir og eftir þurrkun.

Bakki nr.	Fyrir þurrkun [g]	Eftir þurrkun [g]	Þurrefni [%]
1	350,5	35,6	10,2
2	349,3	35,4	10,1
3	345,9	35,1	10,1
4	350,9	35,5	10,1
5	344,4	34,6	10,0
6	347,1	35,2	10,1
Samtals	**2088,1**	**211,4**	**10,1**

Tafla 5 – Hökkuð flök, vigtun fyrir og eftir þurrkun.

Bakki nr.	Hökkuð flök fyrir þurrkun [g]	Hökkuð flök eftir þurrkun [g]	Þurrefni [%]
1	348,4	49,3	14,2
2	344,5	48,7	14,1
3	348,5	48,9	14,0
4	350,6	49,1	14,0
5	350,8	49,2	14,0
6	347,7	48,7	14,0
7	346,4	48,5	14,0
8	350,8	49,4	14,1
9	348,2	49,2	14,1
10	349,4	49,3	14,1
11	349,0	49,0	14,0
12	347,0	47,7	13,7
13	347,0	48,8	14,1
14	350,6	49,0	14,0
Samtals	**4878,9**	**684,8**	**14,0**

Tafla 6 – Hvelja í bitum, vigtun fyrir og eftir þurrkun.

Bakki nr.	Hvelja skorin í bita fyrir þurrkun [g]	Hvelja skotin í bita eftir þurrkun [g]	Þurrefni [%]
1	175	21	12,0
2	175	22	12,6
Samtals	350	43	12,3

3.2.3 Efnamælingar

Sýni voru send í efnamælingu fyrir (Tafla 7 og Tafla 8) og eftir þurrkun. Áhugavert er að sjá mismunandi lit sem og efnasamsetningu hrogna. Sérstaklega hvað viðkemur fitu (Tafla 8). Fituríkustu hrognin (nr. 2 á Mynd 9) voru með fjólubláan lit fyrir þurrkun.

Tafla 7 – Efnasamsetning af haus og flaki fyrir þurrkun.

Sýni	Vatn [%]	Prótein [%]	Fita [%]	Aska [%]	Salt [%]
Hráefni - haus	90,3%	6,6%	1,4%	1,7%	0,9%
Hráefni - flak	86,7%	5,6%	6,0%	1,2%	0,6%

Tafla 8 – Hrogn, efnasamsetning fyrir þurrkun.

Sýni	Vatn [%]	Prótein [%]	Fita [%]	Aska [%]	Salt [%]
Hrogn 1	84,0%	8,1%	2,5%	1,1%	0,8%
Hrogn 2	82,4%	10,9%	5,5%	1,1%	0,8%
Hrogn 3	84,7%	8,4%	3,4%	1,1%	0,8%
Hrogn 4	83,9%	9,2%	2,7%	1,1%	0,8%

Til samanburðar voru þorskflök og þorskprótein sem voru einangruð með pH shift aðferð einnig þurrkuð og efnasamsetning þeirra mæld (Tafla 9). Að lokinni þurrkun hefur hið háa fitumagn í holdinu magnast upp og því orðið hátt í helmingur af þyngd sýnisins.

Áhugavert var að skoða amínósýrusamsetningu hjá hinum mismunandi sýnum. Þar sem grásleppusýnin eru fiturík er magnið hér gefið upp bæði sem per 100g þurrefni annars vegar og 100g prótein hins vegar. Til að auðvelda samanburð er magn amínósýru per 100 g prótein í þorskflaki sett sem 100% og hin skoðuð miðað við það (Tafla 10). Það sem þykir áhugaverðast er með rauðu í töflunni. Sjá má að hrogn eru rík af Cystein+Cystine.

Grásleppuhaus með mikið af Glycini og að haus og hrogn eru rík af Prolin. Ekki síst er áhugavert er að sjá hvað magn af Taurin lækkar í einangruðum þorskpróteinum miðað við þorskflak.

Tafla 9 – Efnasamsetning frostþurrkuð sýni.

Sýni	Vatn [%]	Prótein [%]	Fita [%]	Aska [%]	Salt [%]	Saltlaus aska [%]
Grásleppuflök	1,5	43,2	42,5	8,3	4,07	5,2
Grásleppuhaus	2,5	67,5	8,0	17,1	8,95	10
Hrogn 1	0,3	67,6	12,5	6,5	4,69	4,9
Hrogn 2	0,3	66,5	17,1	5,9	4,17	4,7
Hrogn 3	0,3	65,1	16,3	6,7	4,74	4,9
Hrogn 4	0,3	66,9	14,4	6,4	4,69	4,9
Þorskflök	10,9	88	-	5,4	0,62	0
Einangruð prótein	4	95,5	-	1,3	1,11	0

Tafla 10 – Amínósýrusamsetning sýna. (*Hlutfall m.v. að Þorskflak sé = 1).

Sýni	Amínósýra	Magn [g/100g þurrefni]	Magn [g/100g prótein]	Hlutfall* [%]
Þorskflak	Alanin	4,8	5,5	100
Þorskprótein	Alanin	5,7	5,9	96
Grásleppuflök	Alanin	2,3	5,4	106
Grásleppuhaus	Alanin	4,5	6,6	122
Grásleppuhrogn	Alanin	3,8	5,7	98
Þorskflak	Arginin	5,1	5,8	100
Þorskprótein	Arginin	6,4	6,6	102
Grásleppuflök	Arginin	2,4	5,6	106
Grásleppuhaus	Arginin	4,4	6,5	114
Grásleppuhrogn	Arginin	3,7	5,5	90
Þorskflak	Asparagínsäure	8,4	9,5	100
Þorskprótein	Asparagínsäure	10,4	10,8	101
Grásleppuflök	Asparagínsäure	3,4	7,9	90
Grásleppuhaus	Asparagínsäure	5,2	7,8	82
Grásleppuhrogn	Asparagínsäure	5,7	8,6	84
Þorskflak	Cystein +Cystine	0,9	1,0	100
Þorskprótein	Cystein +Cystine	1,1	1,2	98
Grásleppuflök	Cystein +Cystine	0,4	0,8	87
Grásleppuhaus	Cystein +Cystine	0,5	0,7	72
Grásleppuhrogn	Cystein +Cystine	1,4	2,2	193

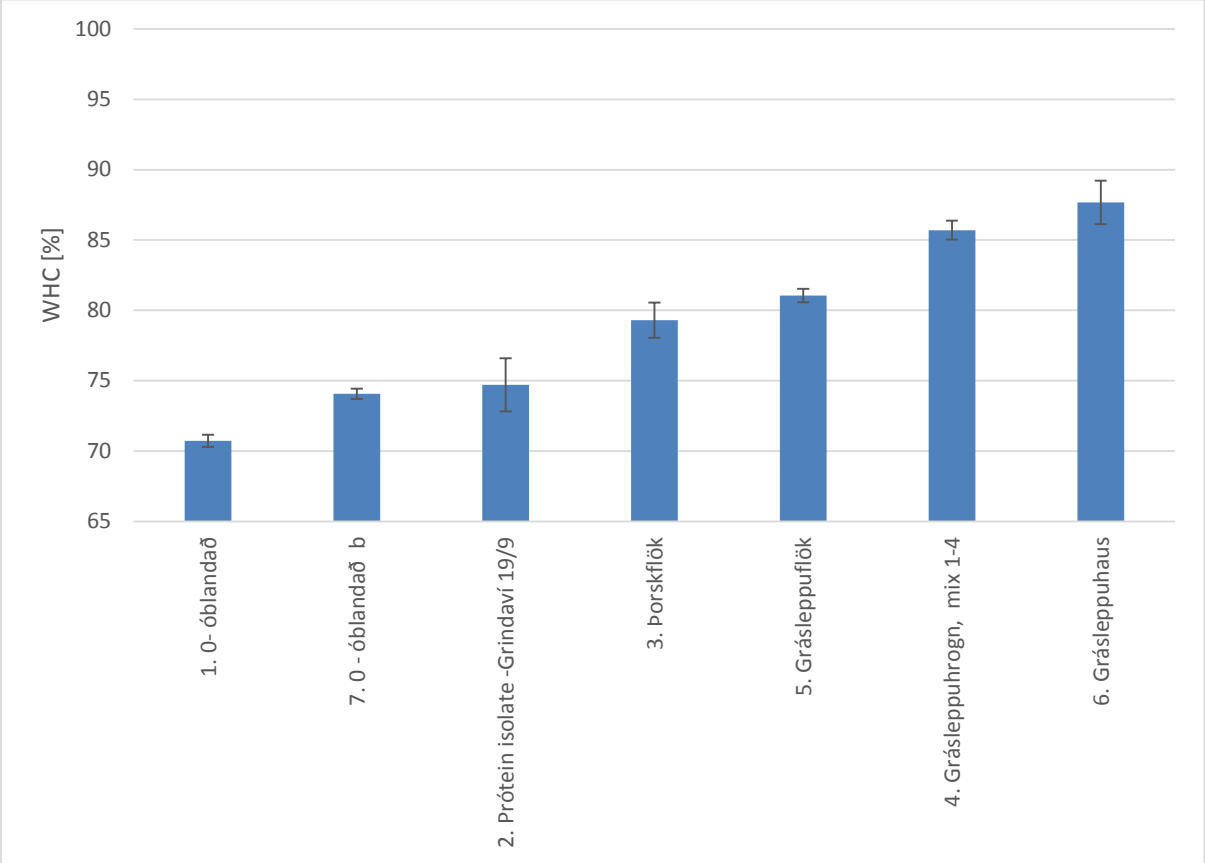
Sýni	Amínósýra	Magn [g/100g þurrefni]	Magn [g/100g prótein]	Hlutfall* [%]
Þorskflak	Glutaminsäure (E620)	11,9	13,5	100
Þorskprótein	Glutaminsäure (E620)	15,3	15,9	105
Grásleppuflök	Glutaminsäure (E620)	4,9	11,4	91
Grásleppuhaus	Glutaminsäure (E620)	7,3	10,8	81
Grásleppuhrogn	Glutaminsäure (E620)	7,6	11,4	79
Þorskflak	Glycin	3,6	4,1	100
Þorskprótein	Glycin	3,6	3,7	81
Grásleppuflök	Glycin	3,2	7,3	193
Grásleppuhaus	Glycin	8,7	12,8	314
Grásleppuhrogn	Glycin	2,2	3,2	73
Þorskflak	Histidin	1,7	1,9	100
Þorskprótein	Histidin	2,1	2,2	100
Grásleppuflök	Histidin	0,7	1,7	95
Grásleppuhaus	Histidin	1,1	1,6	84
Grásleppuhrogn	Histidin	1,8	2,8	133
Þorskflak	Isoleucin	3,6	4,0	100
Þorskprótein	Isoleucin	4,5	4,7	104
Grásleppuflök	Isoleucin	1,4	3,3	88
Grásleppuhaus	Isoleucin	1,8	2,7	67
Grásleppuhrogn	Isoleucin	3,3	5,0	116
Þorskflak	Leucin	6,5	7,4	100
Þorskprótein	Leucin	8,2	8,5	103
Grásleppuflök	Leucin	2,6	6,1	90
Grásleppuhaus	Leucin	3,5	5,2	71
Grásleppuhrogn	Leucin	5,9	8,8	112
Þorskflak	Lysin	7,7	8,8	100
Þorskprótein	Lysin	9,6	9,9	101
Grásleppuflök	Lysin	2,7	6,3	79
Grásleppuhaus	Lysin	3,7	5,4	63
Grásleppuhrogn	Lysin	4,6	6,9	74
Þorskflak	Methionin	2,6	3,0	100
Þorskprótein	Methionin	3,3	3,4	103
Grásleppuflök	Methionin	0,9	2,2	80
Grásleppuhaus	Methionin	1,3	2,0	66
Grásleppuhrogn	Methionin	1,6	2,4	75
Þorskflak	Ornithin	<0.01		
Þorskprótein	Ornithin	<0.01		
Grásleppuflök	Ornithin	<0.01		
Grásleppuhaus	Ornithin	<0.01		
Grásleppuhrogn	Ornithin	<0.01		

Sýni	Amínósýra	Magn [g/100g þurrefni]	Magn [g/100g prótein]	Hlutfall* [%]
Þorskflak	Phenylalanin	3,2	3,7	100
Þorskprótein	Phenylalanin	3,7	3,8	94
Grásleppuflök	Phenylalanin	1,3	3,1	91
Grásleppuhaus	Phenylalanin	2,0	3,0	82
Grásleppuhrogn	Phenylalanin	2,6	3,8	98
Þorskflak	Prolin	2,9	3,3	100
Þorskprótein	Prolin	3,5	3,6	99
Grásleppuflök	Prolin	1,9	4,4	146
Grásleppuhaus	Prolin	4,7	6,9	214
Grásleppuhrogn	Prolin	3,4	5,2	149
Þorskflak	Serin	3,6	4,1	100
Þorskprótein	Serin	4,4	4,6	100
Grásleppuflök	Serin	1,8	4,2	110
Grásleppuhaus	Serin	3,7	5,5	135
Grásleppuhrogn	Serin	4,3	6,5	148
Þorskflak	Taurin	0,7	0,7	100
Þorskprótein	Taurin	0,2	0,2	27
Grásleppuflök	Taurin	0,8	1,9	277
Grásleppuhaus	Taurin	1,0	1,5	201
Grásleppuhrogn	Taurin	0,6	0,9	113
Þorskflak	Threonin	3,6	4,1	100
Þorskprótein	Threonin	4,6	4,8	103
Grásleppuflök	Threonin	1,6	3,7	97
Grásleppuhaus	Threonin	2,5	3,7	91
Grásleppuhrogn	Threonin	3,5	5,2	119
Þorskflak	Tryptophan	0,9	1,1	100
Þorskprótein	Tryptophan	1,2	1,2	101
Grásleppuflök	Tryptophan	0,4	0,8	84
Grásleppuhaus	Tryptophan	0,4	0,6	55
Grásleppuhrogn	Tryptophan	0,8	1,3	112
Þorskflak	Tyrosin	3,0	3,4	100
Þorskprótein	Tyrosin	3,9	4,0	106
Grásleppuflök	Tyrosin	1,0	2,3	75
Grásleppuhaus	Tyrosin	1,0	1,5	45
Grásleppuhrogn	Tyrosin	2,7	4,0	111
Þorskflak	Valin	3,9	4,4	100
Þorskprótein	Valin	4,8	5,0	101
Grásleppuflök	Valin	1,7	3,9	96
Grásleppuhaus	Valin	2,5	3,7	85
Grásleppuhrogn	Valin	3,9	5,9	125

3.2.4 Vinnslueiginleikar

Vatnsheldni (Water holding capacity)

Grásleppuflök sýndu betri vatnsbindieiginleika en þurrkuð þorskflök, þó var munurinn ekki mikill (Mynd 19). Er það sérstaklega áhugavert í ljósi hins háa fituinnihald grásleppunnar. Best vatnsheldni reyndist vera í sýnum úr grásleppuhaus. Ekki má gleymast að saltinnihald sýna var einnig mismunandi og þar innihélt grásleppuhausinn hæst saltmagn. Það hefur einnig veruleg áhrif. Þar sem ekki tókst að einangra próteinin eins og upphaflega stóð til er þó erfitt að greina hvað hefur hér mest áhrif. Eiginleikar próteina eða önnur innihaldsefni.



Mynd 19 – Vatnsheldni þurrkaðra grásleppupróteina og þorskpróteina.

Olíubinding (OBC)

Olíubinding var reiknuð út bæði ber gramm sýni og á hvert gramm próteins í sýni. Sem áður kemur hausinn mjög vel út (Tafla 11).

Tafla 11 – Olíubinding sýna og próteina.

Sýni	OBC [g olía /g sýni]	OBC [g olía /g prótein]
Grásleppuflök	2,1 ± 0,1	4,9 ± 0,1
Grásleppuhaus	5,4 ± 0,5	8,0 ± 0,7
Hrogn	3,0 ± 0,0	4,5 ± 0,0
Þorskflak	3,4 ± 0,1	3,5 ± 0,1
Þorskprótein	2,9 ± 0,1	3,3 ± 0,1

Ýruhæfni (EC)

Ýruhæfni sýna reyndist vera mikill – svo mikill að hann sprengi skala þess tækjabúnað sem var notaður til að mæla eiginleika próteinanna og fékkst því ekki út mælanlegt gildi. Því miður reyndist ekki hægt að fá annan tækjabúnað (smærri ýrunarbúnað) en niðurstöðurnar benda til mikillar ýruhæfni grásleppupróteina.

Ýrustöðugleiki (ES)

Vel gekk að mæla ýrustöðugleika. Sýni skiptust í tvo hópa. Þorskpróteinmassi myndaði ekki ýru og skildi sig strax en þorskflak, haus, hold og hrogn höfðu svo mikinn ýrustöðugleika að eftir 15 mínútur höfðu sýni ekki skilið sig og ýrustöðugleiki því 100%. Jafnvel þó sýni væru látin standa upp í klukkustund höfðu þau ekki skilið sig.

Leysanleiki

Sýni reyndust ekki hafa mikinn leysanleika í vatni enda er sýrustig þess nálægt sama sýrustigi og er náttúrulega til staðar í vefjum. Hrognin sýndu hins vegar þó nokkurn leysanleika (Tafla 12).

Tafla 12 – Vatnsleysanleiki próteina.

Sýni	Duft [g]	Vatn [g]	Prótein [%]	Prótein [g/100g]	Prótein í lausn [%]	Próteinleysan-leiki [%]
Grásleppuflök	2,03	190	43,2	0,45	0,1	22
Grásleppuhaus	2,12	198	67,5	1,43	0,2	28
Hrogn	2,01	190	66,9	1,34	0,5	71
Þorskflak	2,04	190	88,0	1,80	0,3	32
Þorskprótein	2,01	190	95,5	1,92	0,1	10

3.3 Ályktanir á eiginleikum þurrkaðrar grásleppu

Niðurstöður benda til að grásleppuprótein hafi góða vinnslueiginleika – meðal annars með góða vatnsbindieiginleika – sem aftur skapaði vandamáli í verkefninu þar sem ekki tókst að einangra prótein frá öðrum hlutum fisksins. Þar gæti hin mikla olíubinding einnig haft áhrif. Hin þurrkuðu sýni voru hins vegar ekki með góða skynmatseiginleika, voru bæði dökk á lit og með sterka lykt.

4 Vatnsrofin grásleppuprótein

Innan verkefnisins voru gerðar nokkrar tilraunir við að vatnsrjúfa grásleppuprótein og lífvirknieiginleikar þeirra kannaðir. Sú staðreynd að illa gekk að einangra prótein úr mismunandi hlutum gerði vinnuna erfiða. Ýtarlegar skýrslur voru gerðar um tvær af þessum tilraunum og má finna þær í viðauka. Hér á eftir að greint frá helstu niðurstöðum í hverri tilraun. Varðandi framkvæmd og mæliaðferðir er vísað í skýrslur í *viðaukum* II&III.

4.1 Beint vatnsrof

Þar sem ekki tókst að fella prótein út í nægjanlegu magni til að nýta í surimi (verkþáttur 2) eða þurrka einangruð prótein beint til að nota í afurðir var vinna í verkþætti 3 – Þróun þurrkaðra próteinafurða einungis vatnsrof próteina með ensímum. Prófað var að vatnsrjúfa beint hausa og flappa/þunnildi úr grásleppu með Protamex frá Novozymes og Protease M frá Amano. Hausarnir og flappar voru við pH 7 og Protamex sem er virkt við það sýrustig var bætt beint í lausnina. Aftur á móti þurfti að lækka sýrustigið áður en að Protease M var bætt í lausnina til að virkni ensímsins væri sem mest eða niður í um pH 5,5. Nánari verklýsingu fyrir vatnsrof og mælingu á stigi vatnsrofs (Degree of hydrolysis) má sjá í *viðauka*.

4.1.1 Efnasamsetning

Efnasamsetning sýnir að hausar eru fituríkari en flappar, að öðru leiti var ekki mikill munur á efnainnihaldi (Tafla 13). Hið háa próteinininihald í sýni úr flöppum vekur einnig athygli. Þar virðist einhver mælivilla hafa orðið þar sem samanlagt efnainnihald er óeðlilega hátt.

Tafla 13 – Efnasamsetning vatnsrofinna grásleppuhluta.

	Vatn [%]	Prótein [%]	Fita [%]	Aska [%]	Salt [%]
Flappar + Protamex	3,3	97,1	0,31	9,4	7,6
Haus + Protamex	3,6	81,4	1,37	14,7	10,9
Haus + Protease M	4,0	85,7	2,13	9,5	6,7

4.1.2 Lífvirkni

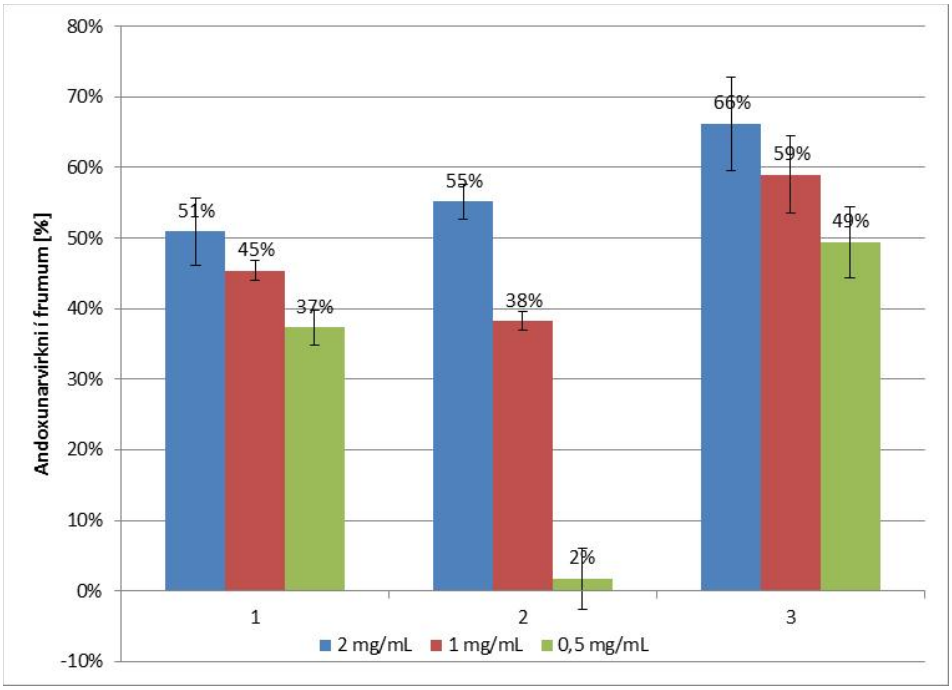
Andoxunareiginleikar sýna voru mældir *in vitro* (Tafla 14). Niðurstöður eru svipaðar því sem fengist hefur fyrir önnur fiskpróteinsýni brotin niður með ensímum fyrir DPPH og Járnbindieiginleika (e. Metal chelating ability) en lakari fyrir ORAC og afoxunarhæfni (e. Reducing power).

Tafla 14 – Andoxunareiginleikar grásleppusýna.

Sýni	ORAC*	DPPH [%]	Járnbindi-eiginleikar [%]	Afoxunar-hæfni**
Flappar + Protamex	230 ± 11	39,6 ± 1,8	22,7 ± 0,7	3,4 ± 0,0
Haus + Protamex	301 ± 15	29,6 ± 0,2	59,8 ± 1,7	5,1 ± 0,0
Haus + Protease M	336 ± 16	32,6 ± 0,3	58,3 ± 0,2	4,9 ± 0,1

*µmol TE/g prótein)
** Ascorbic acid equivalent mg/g prótein

Einnig voru andoxunareiginleikar mældir í frumumódeli (Mynd 20). Mælingar í frumumódeli gefa frekari upplýsingar um hvort efnin hafi andoxandi eiginleika í líkamanum. Þau gildi eru hins vegar hærri en við höfum fengið fyrir önnur niðurbrotin fiskprótein, sérstaklega þau sem voru unnin úr haus frosinnar grásleppu með Protamex (sýni 3 með 66% á Mynd 20). Þær niðurstöður eru því verulega spennandi enda er þetta hráefni sem er ekkert nýtt í dag.



Mynd 20 – Andoxunarvirkni vatnsrofinna próteina úr grásleppu mælt í frumumódeli. 1: Grásleppuþunnildi + Protamex, 2: Grásleppuhaus + Protease M, 3: Grásleppuhaus + Protamex.

Blóðþrýstingslækkandi eiginleikar voru mældir sem hindrun á „Angiotensin coverting enzyme“ (ACE) (Tafla 15). Frekar lág lífvirkni fékkst fyrir vatnsrofin haus og afskurð (þunnildi). Því lægra sem IC_{50} gildi er því virkari er sýnið. Svipað og fyrir andoxun mælt í frumumódeli eru það peptíð unnin úr grásleppuhaus með Protamex sem sýna mesta ACE hindravirkni.

Tafla 15 – Blóðþrýstingslækkandi eiginleikar – ACE hindravirkni.

Sýni	IC_{50} gildi ± staðalfrávik
Grásleppuþunnildi + Protamex	28,1 ± 0,2 mg/ml
Grásleppuhaus + Protease M	19,3 ± 0,3 mg/ml
Grásleppuhaus + Protamex	5,8 ± 0,3 mg/ml

4.1.3 Ályktanir úr mælingum á vatnsrofnum afskurði og haus.

Áhugavert hvað sýni úr haus koma betur út en sýni úr afskurði. Frekari mælingar voru gerða á hausum sbr. Vatnsrof með andoxunarefnum bls. 32.

4.2 Einangruð prótein vatnsrofin

Prótein sem voru einangruð úr grásleppuflökum sbr. kafla 2.2.3 bls. 11 voru vatnsrofin.

4.2.1 Einangruð prótein vatnsrofin með Protamex eða Protease M.

Hráefni var gert að einsleitri lausn með hakkara (Dynamic MF 2000), sýrustig og hitastig mælt og lausnin sett í hitaskáp (43°C). Eftir að lausnin hafði náð réttu hitastigi (40°C) var ensím (Protamex eða Protease M "Amino") sett út í lausnina. Eftir 120 mínútur voru ensímin afvirkjuð með hitun í 90°C í 30 mínútur, síðan var lausnin sett á ís og kæld niður. Loks var lausnin skilvinduð og leysanlegi hlutinn frystur og frostþurrkaður. Einangrun prótein voru fryst við sýrustigið 5,5 og var því ekki breytt áður en að Protease M var bætt í lausnina (virkni Protease M er mest við pH 5,5 og hitatigið 45-55°C (Protease M "Amino")) en hinsvegar var sýrustig hækkað áður en að Protamex er bætt í lausnina. *Sjá* einnig verklýsingu í *viðauka.*

4.2.2 Lífvirkni vatnsrofinna einangraðra próteina.

Stig vatnsrof var mun hærra þegar unnið var með Protease M miðað við Protamex (Tafla 16). Virkni Protease M er yfirleitt hærri en þessi mikli munur er meiri en gert var ráð fyrir. Lífvirkni er hér hærri en þegar unnið var með prótein sem ekki höfðu verið einangruð fyrir vatnsrof úr haus og afskurði. Því miður voru heimtur úr einangrun ekki nægjalegar til að vinna áfram með þessi sýni. Sérstaklega er áhugavert að sjá hina háu ORAC virkni sýna unnin með Protease M sem eru með því hærra sem hefur sést fyrir fiskprótein. Bæði fyrir ORAC og Járnbindieiginleika sést að hið hærra stig vatnsrof (smærri peptíð) skilar sér í hærri lífvirkni.

Tafla 16 – Prósentustig vatnsrofs (degree of hydrolysis, DH [%]) fyrir einangruð grásleppu prótein (Grásl. PI) unnin með Protamex og Protease M. og andoxunareiginleikar þeirra.

Sýni	DH [%]	ORAC*	DPPH [%]	Járnbindi-eiginleikar [%]	ACE IC_{50} [mg/ml]
PI + Protamex	6,7%	399 ± 4	54,3 ± 1,6	33,1 ± 0,7	3,6 ± 0,5
PI + Protease M	25,1%	505 ± 12	55,3 ± 1,3	58,6 ± 0,5	3,8 ± 0,2

*µmol TE/g prótein

4.2.3 Ályktanir úr mælingum á vatnsrofnum einangruðum próteinum.

Lífvirkni sýna unnin úr einangruðum próteinum var með ágætum. Því miður er ekki stefnt á frekari mælingum og þróun á þessum sýni vegna þess hversu lágar heimtur voru úr einangrun.

4.3 Vatnsrof með andoxunarefnum

Við vatnsrof er hætta á oxun sem getur haft áhrif á eiginleika peptíða – bæði jákvæða eiginleika svo sem lífvirkni en einnig neikvæð á neyslueiginleika þeirra. Ákveðið var að prófa að vatnsrjúfa grásleppuhausa með íbættum andoxunarefnum og kanna hvaða áhrif það hefði á mismunandi eiginleika. Grásleppuhausarnir voru valdir því þeir sýndu áhugaverða lífvirkni í fyrrihlutum verkefnisins og þar sem heimtur úr próteineinangrun var ekki nægjanleg til að nýta áfram. Einnig var áveðið að nota ensímið Protease M frá Amano sem er súr próteasi. Eftirfarandi andoxunarefnin voru notuð (skammstöfun innan sviga): kjarni úr bóluþangi (Se-e), rósmarín kjarni (Ro-e) og askorbínsýra eða C-vítamín (Aa). Einnig var viðmiðunarsýni án íblöndunar (Control). Í *viðauka* II er skýrslan „*Bioactive fish protein hydrolysates from Lumpfish heads*“ þar sem farið er nánar í framkvæmd og niðurstöður.

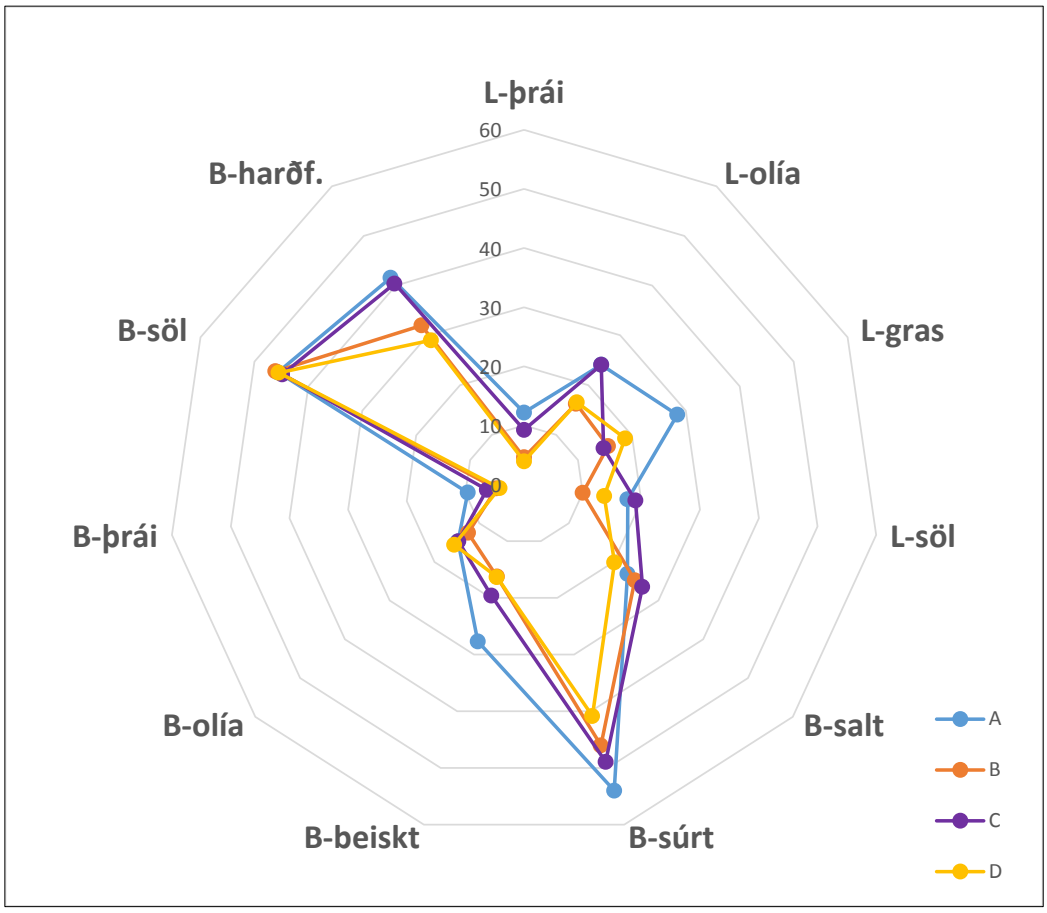
4.3.1 Niðurstöður mælinga á vatnsrofnum hausum ásamt andoxunarefnum

Stig vatnsrofs var um 18% og svipað fyrir öll sýni (Tafla 17). Bóluþangs- og rósmarínkjarnar drógu úr þránun við framleiðslu á meðan C-vítamín virðist frekar hafa hvatt til oxunar frekar en að draga úr. Skynmat sýndi að viðbætur á bóluþangskjarna drógu mest úr þráaeinkennum eins og harðfiskbragði og lykt (Mynd 21).

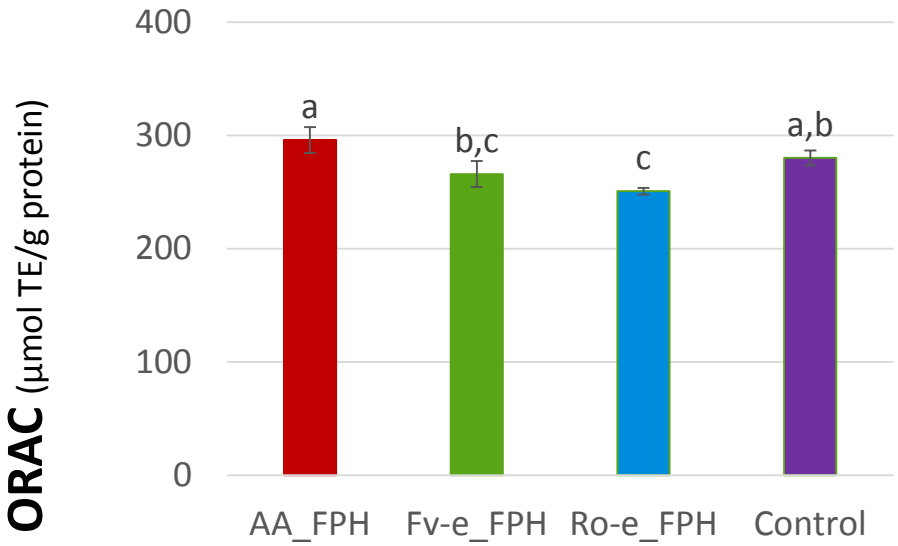
Lífvirkni sýna var einnig mæld. Reyndist andoxunarhæfni þeirra vera svipuð en sýni með bóluþangskjarna komu best út (Mynd 22 til Mynd 24). Einnig var hæfni peptíða til að hindra ensímið ACE kannað (Mynd 25). Viðmiðunarsýni sýndi enga hindrun en af hinum var sýni með bóluþangskjarna virkast.

Tafla 17 – Vatnsrofsstig (DH) og oxun vatnsrofinna grásleppupróteina úr haus með og án andoxunarefna.

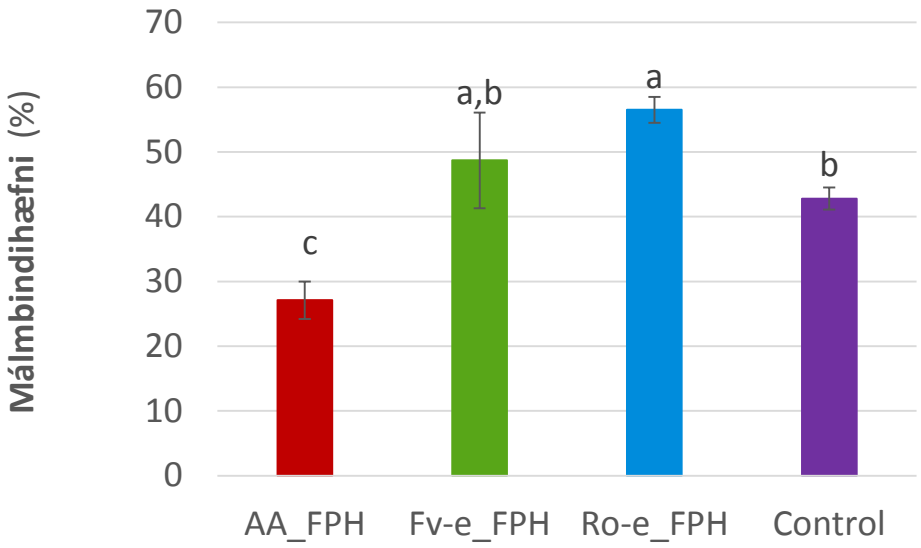
Sýni	Stig vatnsrofs [%]	TBARS (nmol MDA/kg fita)
C-vítamín	18,4	14,1
Bóluþang	16,0	8,4
Rósmarín	18,4	6,6
Viðmið	18,1	14,1



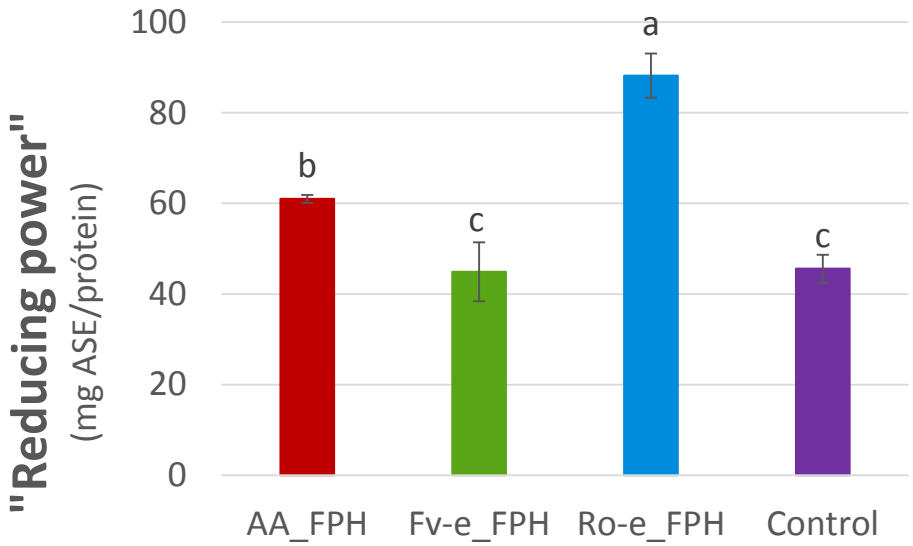
Mynd 21 - Meðaltöl skynmatsþátta fyrir hópa A: C-vítamín, B: Bóluþangskjarni, C: Rósmarínkjarni og D: viðmiðunarsýni. B- => bragð, L- => lykt.



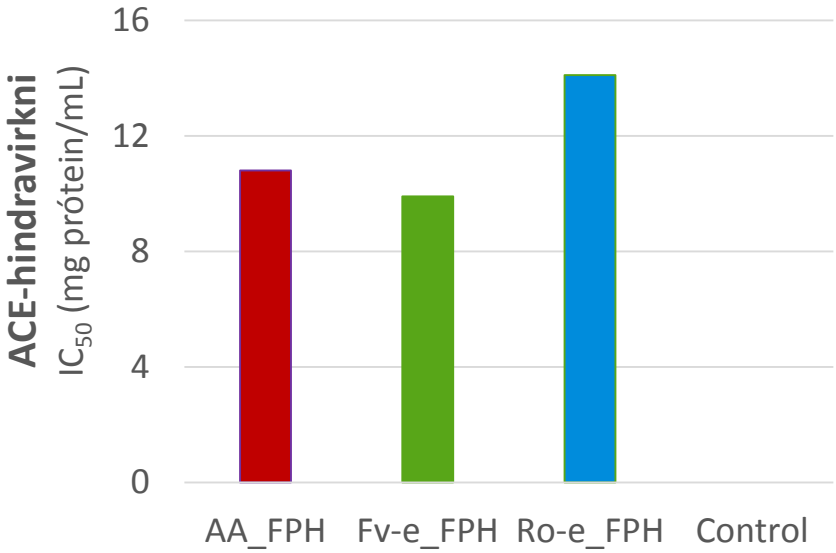
Mynd 22 – ORAC gildi fyrir peptíð unnin úr grásleppuhausum með AA: C-vítamín, Fv-e: Bóluþangskjarni, Ro-e: Rósmarínkjarni og Control: viðmiðunarsýni. Ekki var marktækur munur á milli gilda með sama bókstaf ($p<0,05$).



Mynd 23 – Málmbindigeta peptíða unnin úr grásleppuhausum með AA: C-vítamín, Fv-e: Bóluþangskjarni, Ro-e: Rósmarínkjarni og Control: viðmiðunarsýni. Ekki var marktækur munur á milli gilda með sama bókstaf ($p<0,05$).



Mynd 24 – Andoxunarhæfni peptíða unnin úr grásleppuhausum með AA: C-vítamín, Fv-e: Bóluþangskjarni, Ro-e: Rósmarínkjarni og Control: viðmiðunarsýni. Ekki var marktækur munur á milli gilda með sama bókstaf ($p<0,05$).



Mynd 25 – ACE hindravirkni peptíða unnin úr grásleppuhausum með AA: C-vítamín, Fv-e: Bóluþangskjarni, Ro-e: Rósmarínkjarni og Control: viðmiðunarsýni (engin virkni).

4.4 Frostþurrkuð grásleppa vatnsrofin

Frostþurrkuð prótein úr grásleppu reyndust vera fiturík eins og áður hefur komið fram. Ákveðið var að kanna eiginleika peptíða unnin úr frostþurrkuðu hráefni, hver lífvirkni þeirra væri og hvort hægt væri að lækka fitumagn við vinnsluna. Þurrkuð flök, hausar og hrogn voru notuð sem hráefni og vatnsrofin með tveimur ensímum Protamex frá Novozymes og Proesease M frá Amano. Protease M var notaður við tvö mismunandi sýrustig, annars vega óbreitt sýrustig sem var um pH 6,3 og hins vegar við pH 5,5. Aðferðafræði eins og áður hefur verið lýst.

4.4.1 Niðurstöður

Lægst stig vatnsrofs fékst fyrir sýni með Protamex miðað við Protease M eins og búist var við þar sem virkni protamex er lægra í hverju grammi (Tafla 18). Áhuga vekur munur á stigi vatnsrof fyrir hrogn með Protease M við mismunandi sýrustig. Virkni ensímsins er meiri við pH um 5,5 heldur en 6,3 en afhverju þessi munur kemur sérstaklega fram fyrir hrogn en ekki önnur sýni er ekki ljóst.

Tafla 18 – Samanburður á vatnsrofshlutfalli grásleppuhluta ásamt pH gildi og hitastig sýna fyrir og eftir hýdrólýsu PEX: Protamex, PRM: Protease M

Sýni	pH fyrir	pH eftir	Hitastig fyrir [°C]	Hitastig eftir [°C]	DH [%]
Flök – PEX	6,34	5,89	33,9	38,3	10,3
Flök – PRM	6,32	5,91	33,5	37,3	21,2
Flök – PRM pH 5.5	5,53	5,47	35,2	39,2	17,2
Hausar – PEX	6,64	6,40	33,7	36,4	10,7
Hausar – PRM	6,65	6,38	33,4	35,6	17,2
Hausar – PRM pH 5.5	5,49	5,72	33,1	38,2	17,8
Hrogn – PEX	6,15	6,08	33,3	35,5	4,3
Hrogn - PRM	6,16	6,07	33,0	35,5	8,7
Hrogn – PRM pH 5.5	5,47	5,49	33,4	38,2	16,8

Efnamælingar

Vel gekk að minnka fituinnihald sýna með vatnsrofi og skilvindun fyrir þurrkun (Tafla 19). Hér er komin möguleg góð leið til að bæta eiginleika duftsins. Ekki var nægnalegt hráefni til að mæla heildarefnasamsetningu fyrir hrogn-sýni að loknu vatnsrofi. Þegar próteininnihald sýnanna er skoðuð (Tafla 19) vekur próteininnihald hrognsýna furðu þar sem próteininnihald lækkar eftir vatnsrof ólíkt hinum sýnunum. Þar sem ekki var ngæjanlegt magn af hráefni til að mæla alla efnasamsetningu er ekki ljóst hvað veldur. Eitt sýni var til í nægjanlegu magni til að mæla frekari efnasamsetningu (Hrogn – PM pH 5,5). Þar kom í ljós hærra saltmagn en í öðrum sýnum. Nauðsynlegt er því að taka niðurstöðum á hrognum með vissum fyrirvara.

Tafla 19 – Efnasamsetning sýna fyrir og eftir vatnsrof. PEX: Protamex, PRM: Protease M

Sýni	Vatn [%]	Prótein [%]	Fita [%]	Salt [%]
Grásleppuflök - duft	**1,5**	**43,2**	**42,5**	**4,1**
Flak - PEX	7,8	70,2	4,5	11,3
Flak - PM	9,1	68,1	6,7	10,5
Flak - PM pH 5,5	7,6	69,4	2,2	11,4
Grásleppuhaus - Duft	**2,5**	**67,5**	**8,0**	**9,0**
Hausar - PEX	7,6	74,2	0,5	13,4
Hausar - PM	7,8	73,8	4,7	13,7
Hausar - PM pH 5,5	5,1	71,7	5,4	13,7
Hrogn - duft	**0,3**	**66,5**	**15,1**	**4,6**
Hrogn - PEX	-	48,4	-	-
Hrogn - PM	-	42,1	-	-
Hrogn - PM pH 5,5	-	57,4	2,3	17,2

Lífvirkni

Tafla 20 - Tafla 22 sýna niðurstöður lífvirknimælinga. Aðferðafræði og hrágögn má sjá í Viðauka III. Einstaklega er áhugavert að sjá hversu háa andoxunarvirkni mælt sem ORAC fæst úr vatnsrofnum hrognum.

Tafla 20 – Lífvirkni peptíða unnum úr frostþurrkuðum grásleppuflökum og hausum. PEX: Protamex, PRM: Protease M

Sýni	ORAC [TE/g prótein]	Afoxunarhæfni [„Ascorbic acid equivalent mg/g protein“]
Flök – PEX	378	20,8
Flök – PRM	379	23,6
Flök – PRM-5,5	373	26,5
Hausar – PEX	211	13,6
Hausar – PRM	269	14,0
Hausar – PRM -5,5	324	16,9
Hrogn – PEX	491	22,5
Hrogn - PRM	518	42,6
Hrogn – PRM – 5,5	603	19,8

Járnbindigeta var mæld við misháan styrk. Hér fæst mest virkni fyrir hrognin eins og fyrir ORAC og afoxunarhæfni í síðust töflu.

Tafla 21 – Járnbindigeta peptíða unnum úr frostþurrkuðum grásleppuflökum og hausum. PEX: Protamex, PRM: Protease M

Sýni	1 mg/ml	5 mg/ml	10 mg/ml
Flök – PEX		24,6	37,8
Flök – PRM		24,2	41,8
Flök – PRM - 5,5		21,6	45,1
Hausar – PEX		24,0	34,7
Hausar – PRM		26,7	32,9
Hausar – PRM - 5,5		18,3	25,1
Hrogn – PEX	83,0	97,7	
Hrogn - PRM	93,0	98,2	
Hrogn – PRM – 5,5	70,3	97,6	

Andoxunarhæfni mæld sem DPPH reyndist hins vegar vera einna lægst í hrognum – svipaðar niðurstöður hafa komið fram fyrir önnur sýni – önnur efnabygging skilar háum gildum þar. Einnig þarf að hafa í huga að hér eru sýni leyst upp í etanóli en við aðrar lífvirknimælingar er notast við vatnslausnir.

Tafla 22 – DPPH peptíða unnum úr frostþurrkuðum grásleppuflökum og hausum. PEX: Protamex, PM: Protease M

Hráefni	Ensím	10 mg/mL	5 mg/mL	1 mg/mL
Flak	**Pex**	97,7	97,1	61,4
Flak	**PM**	98,5	97,8	69,5
Flak	**PM 5,5**	97,8	98,4	60,9
Hausar	**Pex**	95,7	88,7	33,6
Hausar	**PM**	96,2	90,8	36,1
Hausar	**PM 5,5**	97,7	75,0	28,3
Hrogn	**PEX**	90,4	63,5	28,8
Hrogn	**PM**	97,4	74,2	27,9
Hrogn	**PM 5,5**	94,9	64,5	26,6

4.4.2 Ályktarnir – vatnsrof á þurrkuðum sýnum

Við vinnu úr frostþurrkuðum sýnum var notast við skilvindun og þess gætt að fjarlægja eins mikið og hægt var af fitufasa. Gekk það ágætlega.

Einnig var áhugavert hvað hrogn sýndu mikla lífvirkni. Það var ekki eitt af markmiðum verkefnisins að kanna hrogn á þennan hátt, þar sem yfirleitt hafa hrogn verið verðmætasta afurð grásleppuveiða. Verð á mörkuðum hafa þó lækkað á síðustu árum og var ákveðið að kanna hér í lokahluta verkefnisins hvernig það reyndist. Er hér kominn möguleiki til að nýta hrogn á nýjan máta og ekki síðar er hægt að benda á þessar niðurstöður þegar rætt er um hollustu grásleppuhrogna.

4.5 Greiningar á peptíðum

Í þessu hluta verkefnisins var kannað hvort hægt væri að hafa áhrif á lífvirkni og stærð peptíða með því að nota þrjú mismunandi ensím og mislangan tíma. Ekki síst var tilgangurinn að setja upp aðferð til geta greint stærð peptíðanna en eitt af vandamálum við markaðssetningu á vörum með smáum peptíðum er að geta greint stærð þeirra, en hefðbundinn rafdráttur nær ekki að mæla svo smá peptíð eins og þau sem við erum að vinna hér með. Í *viðauka* III má sjá ítarlega skýrslu um þennan þátt verkefnisins.

4.5.1 Lífvirkni

Þrjú mismunandi ensím voru notuð (Tafla 23): Protamex frá Novozymes, Protease M sem er súr próteasi frá Amano og Protease P einnig frá Amano. Ensím voru látin vinna í mislangan tíma og ein eða tvö saman. Lengri tími gefur hærra stig vatnsrofs, sérstaklega fæst mikil munur á milli 1 og 3 klst en minna bætist við þegar vatnsrof er haft í 12 klst. Fyrir lífvirknieiginleikana MC og ORAC fæst hærri lífvirkni með hærra stigi vatnsrofs en öfugt samhengi fæst við DPPH. Fyrri rannsóknir hafa sýnt sama „tendens“. Niðurstöður fyrir CAA og ACE hindrun eru ekki nægjanlega margar til að skoða samhengi milli vatnsrofsstigs og lífvirkni.

4.5.2 Stærðargreining peptíða

Tvær mismunandi aðferðir voru notaðar til að mæla stærð grásleppu-peptíða: „Fast Protein Liquid Chromatography“ (FPLC) og „Reversed Phase - High Performance Liquid Chromatography“ (RP-HPLC). FPLC gefur „grófari“ greiningu þar sem stærstu sameindirnar koma fyrst – fara fljótast í gegnum súlurnar á meðan smærri sameindir koma síðastar – eru lengur að komast í gegnum súluna.

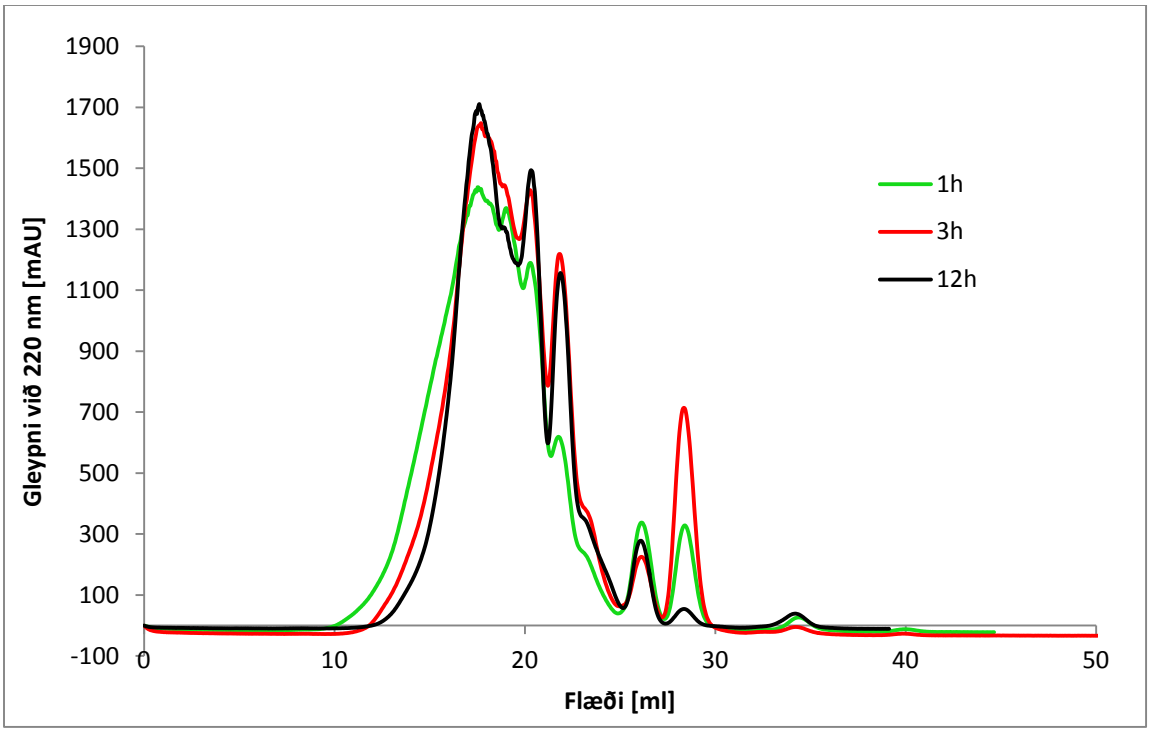
Mynd 26 sýnir dæmi um keyrslu þar sem notað var ensímið Protamex í 1, 3 og 12 klst. Lengri tími skilar smærri peptíðum – saman ber hvað toppur eftir 3 klst er hækkar frá 1 upp í 3 klst eftir 28 ml. Hins vegar kemur á óvart að hann minnkar aftur eftir 12 klst.

Takmarkanir við að nota FPLC við greiningu á stærð er hér greinanleg – til að átta sig betur á stærðinni þarf helst að notast við MS/MS tækni sem Matís hefur því miður ekki yfir að ráða. Mynd 27 sýnir greinilega að eftir 3 klst er Protamex búið að brjóta prótein minna niður heldur en Protease M og P samanber þegar stig vatnsrofs var mælt (Tafla 23).

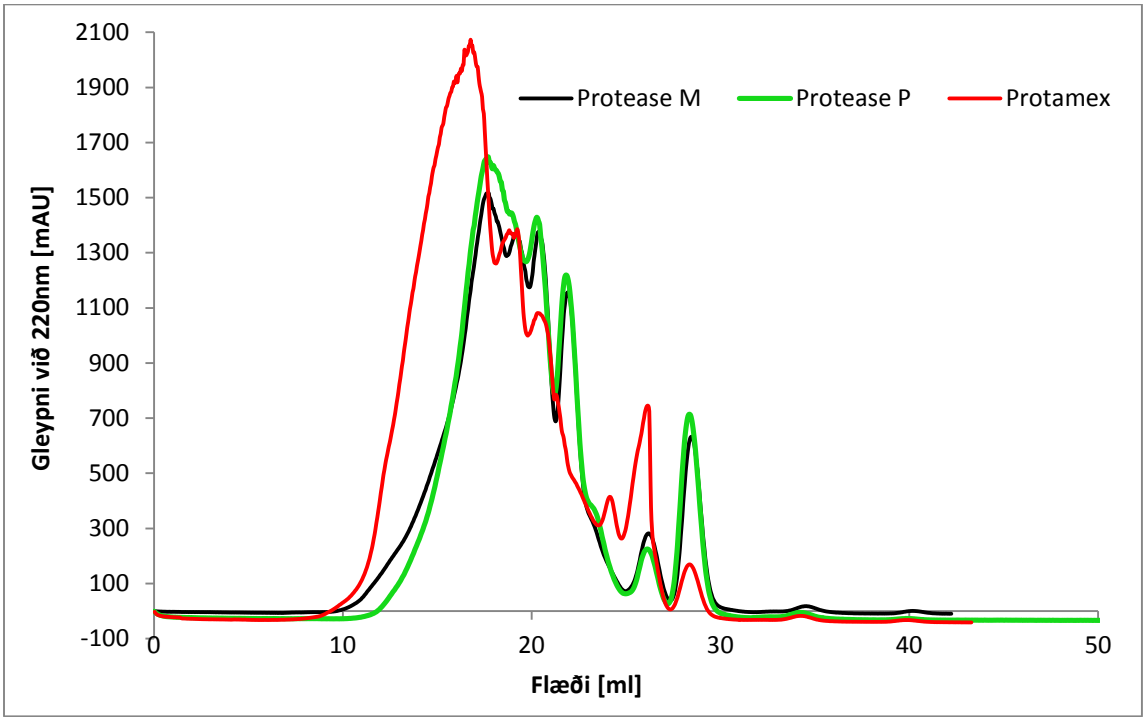
Tafla 23 – Vatnsrofsstig (DH) og lífvirknieiginleikar* nokkurra vatnsrofinna grásleppupróteina. Rauð tala er hæsta gildi fyrir viðkomandi dálk og blá lægsta.

Ensím	DH [%]	MC [%]	DPPH [%]	ORAC gildi	CAA [%]	ACE [IC_{50}]
Protease M í 1klst	16,8	36,7	51,8	362,0	30	7,6
Protease P í 1 klst	23,0	42,8	50,2	464,7		
Protamex í 1 klst	14,7	35,7	52,6	369,1		
Protease M í 3 klst	34,0	24,7	38,4	476,7	47	4,0
Protease P í 3 klst	39,7	32,1	46,7	561,7	31	2,5
Protamex í 3 klst	20,7	27,7	46,3	429,0		
Protease M í 12 klst	48,4	63,6	31,2	527,3	59	4,2
Protease P 12 klst	46,4	88,4	31,9	453,9		
Protamex 12 klst	31,9	28,9	33,8	435,4		
Protease M+Protease P í 2 klst	36,9	33,8	37,2	474,6		
Protease M+Protamex í 2 klst	32,7	31,1	45,1	448,9		
Protease P+Protamex í 12 klst	44,7	58,1	32,1	494,2		

*MC = málbindieiginleikar (Metal chelating activity), DPPH (DPPH radical scavenging capacity), ORAC gildi (Oxygen Radical Absorbance capacity), CAA (Cellular Antioxidant Activity), hindrun á ACE (Antiotensin-converting enzyme inhibitory activity).

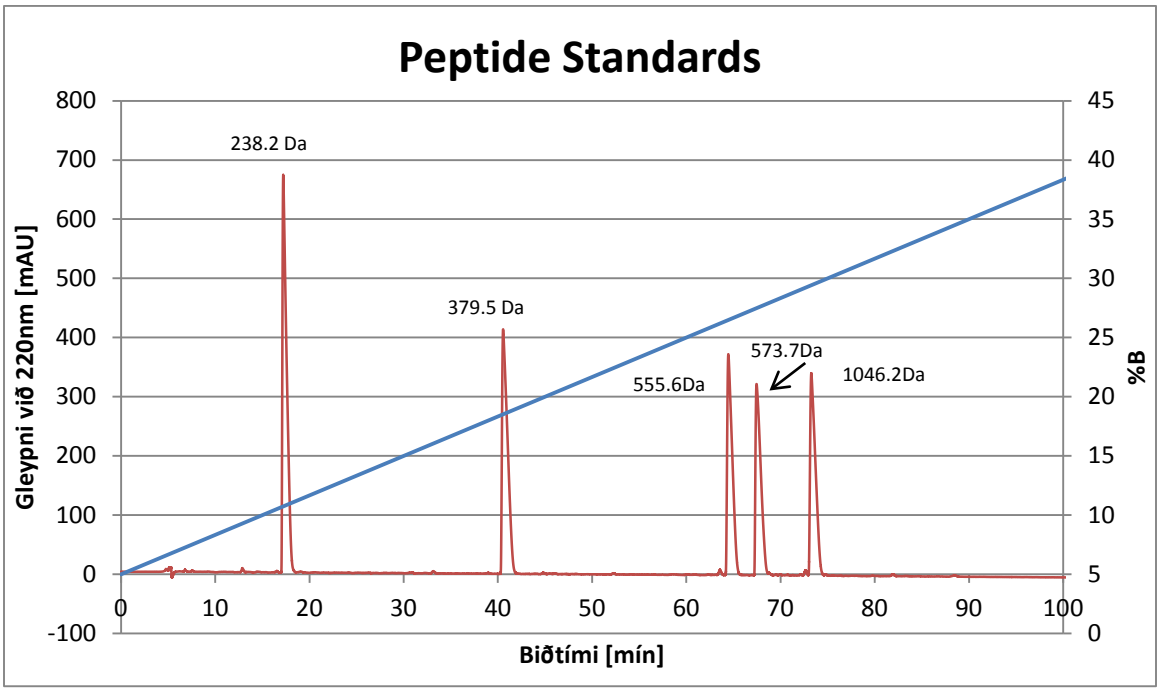


Mynd 26 – Vatnsrofin grásleppupróteinframleidd með Protamex í 1 (græn lína), 3 (rauð lína) og 12 (svört lína) klst keyrð á FPLC tæki.

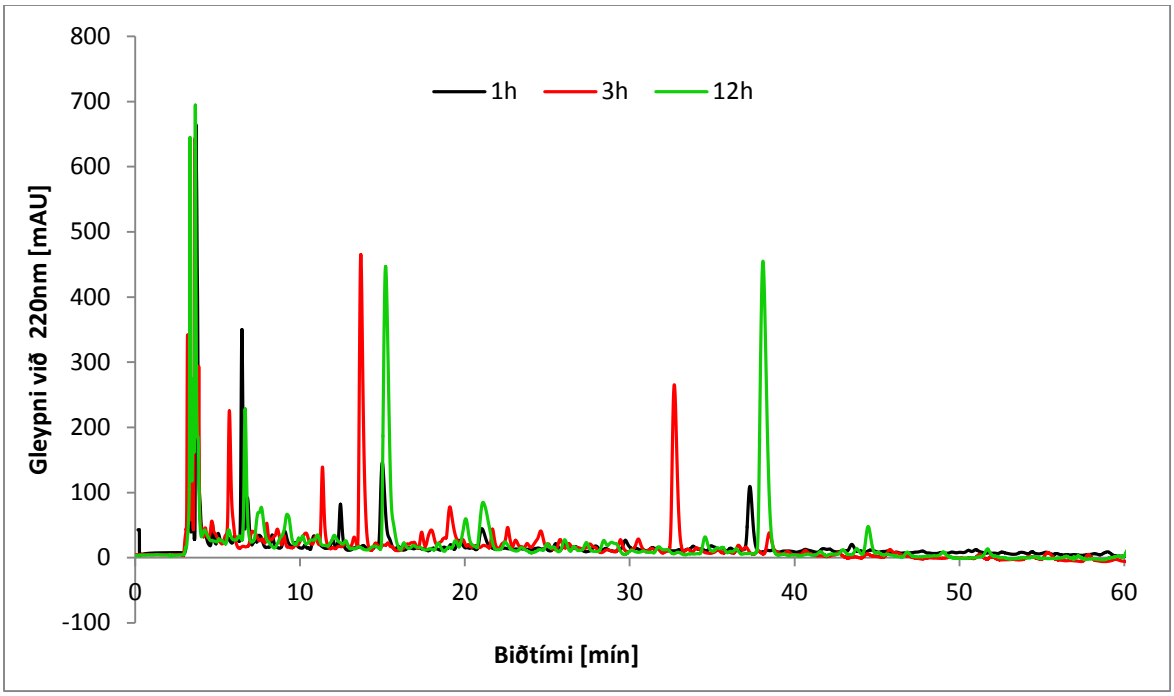


Mynd 27 – Vatnsrofin grásleppupróteinframleidd með Protamex (Rauð lína), Protease P (græn lína) og Protease M (svört lína) keyrð á FPLC tæki.

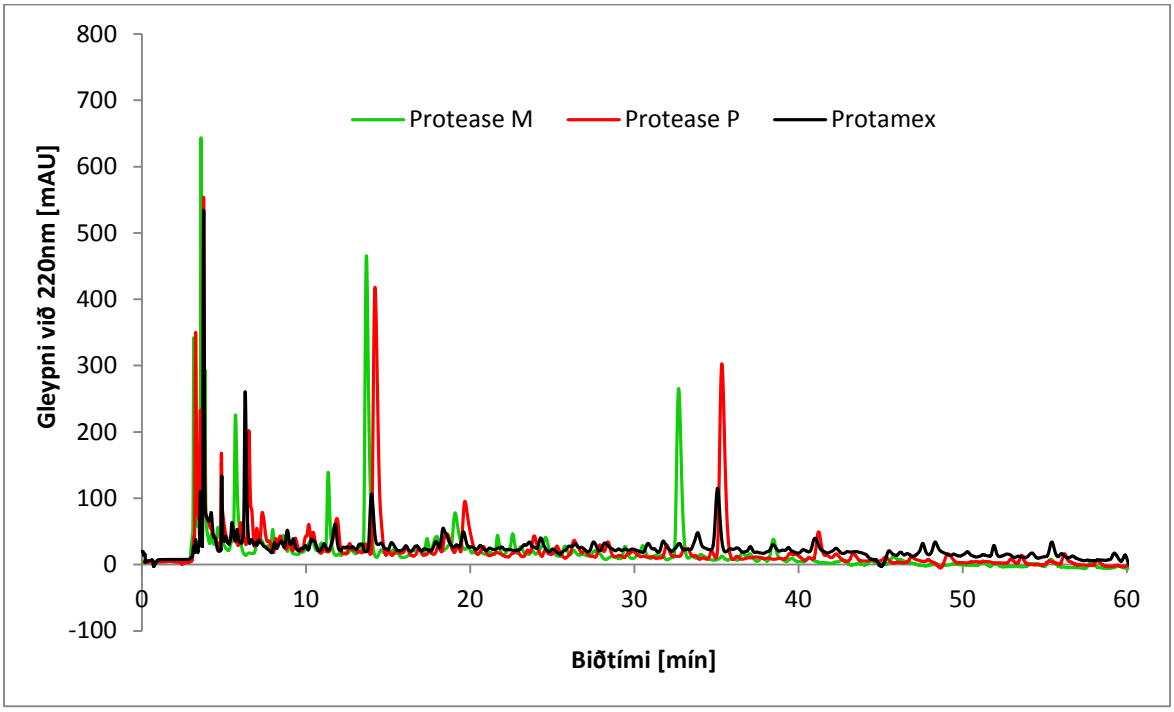
RP-HPLC tækið gefur fínlegri toppa og aðgreinir frekar á milli þeirra mismunandi peptíða sem finna má í sýnunum. Hér hefur hleðsla einnig áhrif á hvenær sameindir koma af súlunni en yfirleitt koma smærri fyrr og stærri eru lengur að fara í gegnum súluna en eins og sjá má á Mynd 28 þá er samband þar á milli ekki línulegt. Ef peptíð er vatnsfælið hefur það mikil áhrif á hvenær þau koma af súlunni, jafnvel frekar heldur en stærð þeirra. Því er ekki greinilegt hvernig mismunandi DH hefur áhrif á hvenær toppar koma þegar mismunandi sýni voru keyrð á RP-HPLC tækinu samanber Mynd 29 og Mynd 30. Þessa tækni er þó gott að nota þegar þátta á sýni niður fyrir frekari greiningar, t.d. þegar sýni eru send til greininga í MS/MS tækjum erlendis en að því er stefnt í framhaldi af þessu verkefni.



Mynd 28 – Peptíð staðlar keyrðir í RP-HPLC. Eftirfarandi peptíð voru notaðir sem staðlar 238,2Da = Gly-Tyr; 379,5Da = Val-Tyr-Val, 555,6Da = Tyr-Gly-Gly-Phe-Leu, 573,7Da = Tyr-Gly-Gly-Phe-Met, 1046,2Da = Asp-Arg-Val-Tyr-Ile-His-Pro-Phe.



Mynd 29 – Vatnsrofin grásleppuprótein framleidd með Protamex í 1 klst (græn lína), 3 klst (rauð lína) og 12 klst (svört lína) keyrð á RP-HPLC tæki.



Mynd 30 – Vatnsrofin grásleppuprótein framleidd með Protamex (svört lína), Protease P (rauð lína) og Protease M (græn lína) keyrð á RP-HPLC tæki.

4.5.3 Ályktanir úr greiningum á peptíðum

Mikilvæg þekking fékst á þáttun og greiningu á peptíðum úr þessum hluta verkefnisins sem reynist mjög mikilvægt fyrir áframhaldandi vinnu.

5 Lokaorð

Þegar lagt var af stað með verkefnið var stefnt að því að einangra prótein út grásleppu með pH shift aðferð en það hefur ekki verið gert áður svo vitað sé. Hin einangruðu prótein átti að nýta í margar gerðir af verðmætum afurðum svo sem surimi og þurrkuð prótein til að nýta sem íblöndunar og/eða fæðubótarefni. Því miður tókst ekki að einangra próteinin og hin upphaflegu markmið verkefnisins náðust því ekki. Framtíðin mun vonandi leiða í ljós hvernig er best að einangra prótein úr grásleppu, þó það hafi ekki tekist hér.

Í verkefninu var unnin mikil þróunarvinna á sviði einangrunar peptíða og þróun á lokaafurðum sem skilaði miklu magni af þekkingu og reynslu sem nýtist áfram bæði til að auka við notkun á grásleppu sem og öðru hráefni sem er ekki nýtt í dag.

6 Þakkir

AVS er þakkað fyrir stuðninginn, samstarfsfólki hjá Matís og ekki síst skynmatshópnum er þökkuð mikil vinna og þrautsegja við að leysa verkefnið.

7 Heimildir

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8 VIÐAUKAR

Viðauki I - Aðferðir

Leysanleiki Biuret aðferð

1. Biuret hvarflausn

Leyst er upp
1,50 g $CuSO_4*5H_2O$ og 6,0 g $NaKC_4H_4O_4*4H_2O$ í 500 ml eimuðu vatni
Bætt er í með hræringu
300 ml. 10% NaOH
Þynnt í 1 lítra með eimuðu vatni

2. Aðferð

1) Flotið er þynnt með vatni, svo próteininnihald mælilausnar verði á bilinu 2-10 mg. Unnið er með tvísýni.
2) Löguð eru eftirfarandi sýni (þrísýni af hverju tvísýni):

	Blankur	Sýni	Staðall
Vatn	0,6 ml	----	----
Próteinlausn	----	0,6 ml	----
BSA – Staðall	----	----	0,6 ml
Biuret-lausn	2,5 ml	2,5 ml	2,5 ml

3) Af staðlinu er löguð tvö sýni 4mg/ml og 6 mg/ml af BSA (sjá staðalkúrfu).
4) Sýnin eru löguð í tilraunaglös og blönduð á Vortex.
5) Þau eru látin standa í 35 mín við stofuhita og þá færð yfir í 4 ml plastkúvettur.
6) Lesin er gleypni lausna við 540 nm skv. leiðbeiningu er fylgja mælitæki.
7) Próteinstyrkur er fundinn út frá samanburði við staðalkúrfu.

3. Útreikningar

Próteinstyrkur er lesinn af staðalkúrfu.

4. Staðalkúrfa

Löguð er BSA- grunnlausn (Bovine Serum Albumin) er leyst 600 mg í 50 ml af vatni. Notuð er 50 ml mæliflaska. Úr þessari lausn eru lagaðar þynningar í tilraunaglös samkvæmt eftirfarandi töflu:

BSA [mg/ml]	Grunnlausn [ml]	Vatn [ml]
1	0,25	2,75
2	0,5	2,5
4	1	2
6	1,5	1,5
8	2	1
10	2,5	0,5

Síðan er haldið áfram samkvæmt lið 2) og unnið er með a.m.k. þrísýni.

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Próteineinangrun með pH shift aðferð

1) Ef unnið er með frosið hráefni er byrjað á því daginn áður að afþýða hann. þ.e hann er settur í nokkra tíma við herbergishita og geymdur síðan yfir nótt í kæli. Daginn eftir er hann tekinn út úr kæli og afþýddur alveg, með því að láta renna á hann vatn (passa að fara alls ekki yfir 10°C).

   Ef fiskurinn er ferskur er farið beint í skref 2.

2) Hveljan, hausinn og sporðurinn er fjarlægður (best er að fjarlægja það á meðan að enn er smá frost í fisknum).

3) Vöðvi, beingarður og mæna vigtuð (fyrir hökkun).

4) Fiskurinn (Vöðvi, mæna og beingarður) hakkaður. **ATH. Passa að taka sýni fyrir próteinmælingu (efnagreiningarmælingu.) áður en að vatn er sett á fiskhakkið.**

5) Hakkið vigtað og 6 hlutum af vatni blandað við hakkið (heildin eru því 7 hlutar).

6) Lausnin gerð einsleit (til að fá hakkið enn smærra).

7) pH mælir stilltur með buffer 7 og 10 (því við förum basa leiðina, þ.e stillum fyrst sýrustigið á um 11)

8) Sýrustig lausnarinnar ákvarðað (þ.e upphafssýrustigið)

9) 2M NaOH vigtað (upphafs massi).

10) Sýrustig hækkað með NaOH í um 11 (passa að fara alls ekki yfir 11,2).

11) 2M NaOH sett aftur á vigtina og massi þess sem fór í lausn ákvarðaður

12) Sía (lausninni hellt í gegnum svartan gúmmídúk á grind)

13) pH mælir stilltur með 7 og 4

14) Sýrustig lækkað niður í milli 5,5 og 5

15) Sía. 4 faldur klútur settur á sigti til að sía vatnið frá próteinmassa.

16) Prótein massi vigtað, 100 -150g sett í hvern poka, lofttæmt, pakkað og fryst í -24°C.

17) Taka sýni fyrir rafdrátt, af hakki (áður en að vatn er sett á það), vatni (sem tekið er af próteinmassa) og próteinmassa

Sample preparation

1) Turn on the water bath and adjust temperature setting to 40-43°C.
2) Fill some plastic bags with tap water and put it into the water bath.
3) Cut fish filets into hand-sized pieces and put them into a blender.
4) Mix the fish pieces for around 5 seconds.
5) Put the mince into a bucket and weight it out (pay attention to use net weight!)
6) Note the dry mass of the sample amount
7) Add 40-43°C preheated water to the fish mince
8) Note the weight of the mixture
9) Mix the solution on the ground with a hand-held blender until it´s homogenize.
10) Calibrate the pH-electrode and note the accuracy of the pH-meter.
11) Adjust the pH of the mince with 2M HCl to wanted value by vigorously stirring the fish mince with a big spoon.
12) Note the used acid/base quantity
13) Fill the ready fish sample into Erlenmeyer flask.
14) Note the weight of every filled Erlenmeyer flask.
15) Adjust water bath to 90°C for later enzyme inactivation

Enzyme hydrolysis

16) Adjust a temperature controlled shaker to 45°C and if necessary mount suitable flask holder on the shaker plate. Consider the circumstances that all Erlenmeyer flasks have the same starting temperature!
17) Put the samples into the shaker and let them swirl until right temperature is reached
18) Calculate the required amount of enzymes out of the protein:enzyme assuming the protein content in the fish sample
19) Weight out the enzymes and note the used amount for each flask.
20) Measure the temperature and pH of each flask and note the values.
21) Change pH-value with 2M HCl or 2M NaOH (for more alkaline conditions) if needed in regard to the wanted conditions.
22) Add the enzymes to the right flasks and let the enzymes work for x -hours (flexible between experiments).
23) Stop the process after x-hours and take notice from pH and temperature

After hydrolysis

24) Put the fish hydrolysates into labeled plastic bags, close it twice for 2 seconds with a heating rod and store it for 30 minutes in the 90°C preheated water bath (depends on samples size).
25) Cool the samples down on ice and in the meantime prepare things for the centrifugation step.
26) Fill the samples into flasks and measure the pH and temperature.
27) Take approximately 50ml of each sample for OPA analysis
28) Pour the samples into centrifugation tubes and balance two with each other (±0.05g).
29) Centrifuge the fish hydrolysates with 10,000rpm for 20min. Use for this step a fresh cooled rotor!
30) Filter the samples after centrifugation through a cheese cloth and collect them in a flask.
31) Spread the filtrate evenly on labeled aluminum boxes and store them at -24°C before freeze drying.

Degree of hydrolysis with OPA method

Chemicals

OPA skv. Nielsen *et al.*, 2001

Chemical	IFL Number
di-Na-tetraborate decahydrate	A1302
SDS (Na-Dodecyl-sulfate)	G4205
OPA (o-phthaldialdehyde 97%)	G3605F
Ethanol	H1040
DTT (Dithiothreitol 99%)	G1620R
Serine (Art. 7769 Merck)	D2026/D2082F

Apparatus

Erlenmeyer flasks: 100ml, 200 ml and 500 ml
Test tubes: 10 ml
A 4-decimal analytical balance
Pipettes: 400 µl, 3 mL and 4 mL
Magnetic stirrer
Whirl mixer
Spectrophotometer at 340 nm

Reagents

The OPA reagent was prepared as follows:

1. 7.620 g di-Na-tetraborate decahydrate and 200 mg SDS were dissolved in 150 ml deionized water – use stirring and heating (approx. 30°C)
2. The reagents were completely dissolved before continuing
3. 160 mg OPA was dissolved in 4 mL ethanol
4. OPA solution was transferred quantitatively to the above mentioned solution by rinsing with deionized water
5. 176 mg DTT was added to the solution by rinsing with deionized water
6. The solution was made up to 200 ml with deionized water

The serine standard was prepared as follows:

7. 50 mg serine was diluted in 500 ml deionized water (0.9516 meqv/L).

The sample solution was prepared as follows:

8. X g sample was dissolved in 100ml deionized water
9. X is 0.1 to 1.0 g sample containing 8% to 80% protein
10. The DH of the sample also influences the amount required.

Procedure

1. All spectrophotometer readings were performed at 340 nm using deionized water as the control
2. Three ml OPA reagents were added to all test tubes
3. Test tubes used for analyzing 1 sample (double determinations were) Standard 4 tubes; Blank 4 tubes; Sample 4 tubes
4. As absorbance changes somewhat with time it is important the samples stand for exactly the same time (2 min) before measuring.
5. The assay was carried out at room temperature.

Standard measuring

6. 400 µl serine standard was added to a test tube (time 0) containing 3 ml OPA reagents and mixed for 6 s.
7. The mixture stood for exactly 2 min before being read at 340 nm in the spectrophotometer.
8. Two standards were measured before the blanks along with sample values.
9. The last 2 standard were measured after having determined all blanks and sample values.
10. The mean of these standards was used for calculations
11. The typical value of the standards was OD about 0.8.

Blank measuring

12. Blanks were prepared from 400 µl deionized water and treated as described above. The typical value of a blank was OD about 0.07.

Sample measuring

13. Samples were prepared from 400 µl sample

Obs. If OD is higher that 1.0 – dilute the samples.

Calculation

Determination of h

$$Serine\text{-}NH_2 = \frac{OD_{sample} - OD_{blank}}{OD_{standard} - OD_{blank}} * 0.9516 \frac{meqv}{L} * 0.1 * \frac{100}{X} * P$$

where

Serine-NH_2: meqv serine NH_2 / g protein
X: g sample
P:protein % in sample
0.1:Sample volume in liter (L)

h is then:

$$h = \frac{Serine\text{-}NH_2 - \beta}{\alpha \ meqv/g\ protein}$$

Where α and β are shown in table 1 for specific raw materials

Table 1 – Value of constants α, β and h_{tot} for different protein raw materials (Adler-Nissen 1986)

Protein	α	β	h_{tot}
Soy	0.970	0.342	7.8
Gluten*	1.00	0.40	8.3
Casein	1.039	0.383	8.2
Whey*	1.00	0.40	8.8
Gelatin	0.796	0.457	11.1
Meat*	7.00	0.40	7.6
Fish*	1.00	0.40	8.6

*When raw material has not been examined, then α and β are estimated to be 1.00 and 0.40 respectively

Determination of DH

$$DH = \frac{h}{h_{tot}} * 100\%$$

Where h_{tot} for specific raw materials is found in table 1.

The method should be used with caution at low DH

Nielsen, P.M., Petersen, D. and Dambmann, C. (2001). Improved method for determining food protein degree of hydrolysis. J. Food Sci. 66: 642-646

VIÐAUKI II

Bioactive fish protein hydrolysates from Lumpfish heads

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Sigrún Mjöll Halldórsdóttir

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1 Introduction

1.1 Lumpfish

Lumpfish (*Cyclopterus lumpus)* are distributed throughout the North Atlantic Ocean. They are mostly underutilized apart from their spawns, which are used to make caviar. Therefore when the spawns have been taken, the rest of the fish is mostly discarded (Freeman, Kasper and Kristmundsson, 2013).

Not many researches have been performed regarding exploitation of Lumpfish. One research on Lumpfish was performed in 1975 where they examined the content and the distribution of lipids in lumpfish, and also the characteristics of the oil and meal by reduction, and the possibility of glue production from the skin. But the results showed that it was impractical using by-product from Lumpfish at that time (Paradis, Ackman, Hingley and Eaton, 1975).

Since then little has been done regarding research in finding a better use of this fish. Lumpfish heads are currently underutilized material and that is why it would be interesting if there would be a way to use it by adding value to it. Preliminary research, performed at Matís ohf. show that hydrolysates from Lumpfish heads tend to have a great deal of bioactivity.

1.2 Natural Antioxidants

Oxidation takes place in every living organism that exploits oxygen from the environment. Because of that the biological system has developed various amounts of antioxidants to protect themselves against the oxidation process (Damodaran, Parkin and Fennema, 2008).

The oxidation process that occurs in food can be slowed down by using antioxidants. They also slow down undesirable changes that can take place because of the oxidation, such as color changes and browning, they can maintain the nutritional value of the food and they can also prevent that bad taste and bad smell are formed in the food (Hudson, 1990).
Natural antioxidants are a more popular choice, when it comes to using antioxidants in food then chemically made antioxidants. The reason is simply because it is what the consumers and society prefer and want, and that is why use of chemically made antioxidants is generally decreasing in the world. Natural antioxidants are considered to be safe even though they have not yet been fully investigated (Frankel, 2007). Researches have also proven that synthetic antioxidants can have harmful effects on consumers' health (Augustyniak, Bartosz, Cipak and

others, 2010). Ascorbic acid, rosemary extract and seaweed extract are an example of highly effective natural antioxidants.

1.2.1 Ascorbic acid

Ascorbic acid is a secondary antioxidant, it is water-soluble and it has multiple functions where it can work as a reducing agent. That means it can slow down the oxidation rate by maintaining metal ions in its higher oxidation state.

It can also work as a metal chelator, meaning that the antioxidant forms a complex with the metal, so it basically cannot do anything or have any effects.

Ascorbic acid can also have pro-oxidant activity. It tends to be rather difficult to predict exact function of it as an antioxidant, but that is because it can show different effects in the same food or biological system. These effects are dependent on what methods and even conditions are used for activity testing (Frankel, 2007).

1.2.2 Rosemary extract

Among herbs, Rosemary is well known for its high antioxidant activity. The extract of it is used in foods because of these strong antioxidant properties, which are related to the phenolic structure, but those phenols have a radical scavenging ability.

Phenolic deterpenes are a primary antioxidant and are commonly found in plants, where they for example function as hormones.

The antioxidant activity is due to Carnosic acid and Carnasol as main components and to Rosemarinic acid as a minor component (Schwarz, 2002; Frankel, 2007).

Antioxidant activity of rosemary *in vitro* is considered to be due to its reducing agent ability and also as a radical scavenger, which prevents the formation of a singlet oxygen and combination to pro-oxidant metal ions (Santos, Shetty and Miglioranza, 2014)

1.2.3 Seaweed extract

In this research the seaweed extract was made with water extraction from the Icelandic brown algae, *Fucus vesiculosus*, which is a brown algae species located in Iceland, and is considered to be a highly effective antioxidant (Wang, Jónsdóttir, Liu, Gu, Kristinsson, Raghavan and Ólafsdóttir, 2012).

Seaweed extract has so called polyphenolic compounds. They consist of a hydroxyl group, and they can donate hydrogen to form a stable phenoxy radical. The antioxidant activity has also been linked with reducing power (Frankel, 2007; Halldórsdóttir, 2013).

Polyphenols found in algae are a good example of highly effective natural antioxidant, especially phlorotannins which can be found in brown seaweed. It can have up to 8 aromatic rings that are interconnected, which explains why seaweed extract is such an excellent antioxidant (Halldórsdóttir, 2013).

1.3 Fish protein hydrolysates (FPH)

Oxidation can be a problem during enzymatic hydrolysis and antioxidant strategies can enhance the quality of fish protein hydrolysates.

Fish protein hydrolysates can be defined as proteins that have been broken down into peptides of various sizes. This can be achieved either with chemical methods where the peptide bonds are broken with either acid, base or with a biological process where enzymes are used to hydrolyze peptide bonds. These peptides that are formed have different physiochemical properties and functional properties then the original protein. The use of enzymes to hydrolyze food proteins triggers an important process to improve or change this physiochemical-, functional- and sensory properties of the original protein (Kristinsson and Rasco, 2000). When using enzymes to hydrolysates fish proteins the conditions are milder, it is easier to control them and it is more environmentally friendly as compared to chemical methods (Halldórsdóttir, 2013).

The functional properties of FPH can be improved by using special enzymes and make sure to choose the right conditions regarding that specific enzyme, such as time, pH and temperature (Liceaga-Gesualdo and Li-Chan, 1999).

Peptides from FPH commonly possess various functional properties such as ACE (angiotensin converting enzyme) inhibitor effects, antimicrobial effects and antioxidant properties (Vercruysse, Van Camp and Amagghie, 2005).

1.4 Bioactivity analysis

Antioxidant properties of FPH can be measured with many types of tests, which can be divided into *in vivo* measurements and *in vitro* measurements. I*n vitro* measurements are for example

DPPH (2,2-diphenyl-1-picrylhydrazyl), ORAC (oxygen radical absorbance capacity), ACE inhibitor analysis, reducing power and metal chelating ability, and are commonly used when measuring antioxidant properties of hydrolyzed peptides (Vermeirssen, Van Camp and Vestraete, 2004).

DPPH measures the ability of a sample to inhibit DPPH radicals, to evaluate its antioxidant ability. DPPH is a stable radical with a purple solution which turns yellow if there is an antioxidant present in the sample. Measurements start as soon as the sample is mixed with the solution, and they are repeated over period of time to see how long it takes the sample to deactivate certain amount of DPPH in the solution, that is, how powerful the sample is as an antioxidant. The test is widely used because of how easy it is to perform (Deng, Cheng and Yang, 2011; Kumar, Ganesan and Rao, 2008).

ORAC analysis measures the ability of a sample to absorb oxygen and calculates antioxidant activity according to a standard, which in most cases is Trolox. Measurements are performed while the oxidation reaction takes place but the reaction is measured with fluorescent. The radical who has these effects on the sample is called peroxy radical, and because of these effects the oxidation takes place (Price, Sanny and Shevlin, 2006).

Reducing power indicates the ability of a sample to reduce charge by donating an electron and by doing so it prevents oxidation (Frankel, 2007). Measurements are done in a spectrophotometer where the sample is compared to standards (Yang, Guo and Yuan, 2008).

The metal chelating ability measures the ability of a sample to form a soluble complex with metal ions that induce oxidation, thereby disabling them (Frankel, 2007). The measurements are carried out in a spectrophotometer and percentage of chelating effects calculated (Gulcin, Buyukokuroglu and Kufrevioglu, 2003).

ACE inhibitor analysis measures the ability of peptides in a sample to inhibit the activity of ACE. This enzyme converts angiotensin I into angiotensin II, which causes the vessel wall to get tighter and so the blood pressure increases. ACE inhibitors prevent that angiotensin I converts into angiotensin II, keeping the blood pressure low (Camp, Smagghe and Vercruysse, 2005; Widmaier, Strang and Raff, 2008).

1.5 Rancidity analysis

There are many types of methods to measure lipid oxidation in food. They can be divided into two groups, one that measures primary oxidative changes and then one group that determines secondary changes that occurs in a system (Akoh and Min, 2002).

Peroxide value (PV) indicates the primary oxidative changes in lipid oxidation. It represents the total hydroperoxide content in the sample and it is one of the most common quality indicators of fats and oils during storage and production.

Thiobarbituric acid reactive substances (TBARS) measures secondary oxidative changes in lipid oxidation. It is the most widely used method to detect oxidative deterioration in food that contains fat (Shahidi and Zhong, 2005).

1.6 Objectives

The objectives of this research was to add value to a underutilized product, by producing bioactive compounds from minced Lumpfish heads, and evaluate if different antioxidants had any effects. Three different antioxidants were used in the process; ascorbic acid, rosemary extract and seaweed extract.

2 Materials and methods

2.1 Chemicals

Borax (di-Na-tetraborate decahydrate) and the antioxidant Acidum Ascorbicum, were bought from Norsk medicinal depot in Oslo, Norway. The seaweed extract (*Fucus vesiculosus* extract) was made in Matís Reykjavík in Iceland and the rosemary extract was purchased from Optimal á Íslandi ehf. in Grindavík, Iceland. The enzyme Protease M was purchased from Amano Enzymes in Nogoya, Japan. SDS (Na-Dodecyl-sulfate), OPA (o-phthaldialdehyde), DTT (Dithiothreitol), L-Serine, BHT (butylated hydroxytoluene), sodium chloride solution, ammonium thiocyanate, chloride solution, TCA (Trichloroacetic acid), Propyl gallate, EDTA (Ethylenediaminetetraacetic acid) and Thiobarbituric acid were all purchased from Sigma Aldrich in St. Louise, USA. Spiritus fortis Ethanolum (96 %) was purchased from Gamla Apótekið in Reykjavík, Iceland.

2.2 Enzyme hydrolysates

To start with a hot water bath (Julabor 33, T-243) was prepared by heating it up to 40°C, where 4 L of tap water were heated. The next step was to collect a sample for rancidity measurements of the minced lumpfish heads. Then 2.7 kg of minced Lumpfish heads and 3.3 kg of hot water were mixed together and homogenized (Dynamic homogenizer) for approximately 2 minutes. The hot water bath was then set to 90°C. When the homogenization had taken place the pH was adjusted to 4.5, which is the optimal conditions of the enzyme, with 5 mL of NaCl and 65 mL of 2.0 M HCl. When the pH had been adjusted samples were collected for rancidity measurements and also chemical analysis.

Then the homogenized solution was divided into 4 equal parts where approximately 1500 mL were put into 4 Erlenmeyer flasks. Antioxidants were then added to the solutions where 0.5 g of Acidum Ascorbicum were measured and mixed with the solution in the first Erlenmeyer flaks. Then 0.5 g of seaweed extract was put in the second flask, and 0.5 g of rosemary extract was added to the third solution. The fourth solution contained no antioxidant. When the antioxidants had been thoroughly mixed together with the solutions, 1.0 g of the enzyme Protease M was added and mixed to all four solutions, and they put in an incubator where the enzyme reaction took place at 42°C and 120 rpm for 2 hours. After 1 hour the position of the flasks were changed in the incubator. After 2 hours the solutions were taken out of the incubator

and poured into plastic bags, they sealed and put in 90°C hot water bath for 15 minutes to stop the enzyme reaction. Thereafter the bags with the solutions were put on ice for 10 minutes to cool them down. After they had cooled down, samples were taken for OPA measurements and rancidity measurements for all four samples. The samples were then poured into centrifuge tubes and centrifuged (Beckman Coulter USA) at 10.000 g and 4°C for 20 minutes. The precipitant was discarded, but the supernatant was poured into aluminum trays and a lid placed over them, which were then put into a freezer at -24°C. When the supernatant had frozen through the lids were taken of the trays and they placed in the freeze drier (Virtis genesis, Midland, Canada) for two days. Thereafter the samples were grinded and samples collected for rancidity, bioactivity analysis, sensory analysis and chemical analysis. The rest of the samples were stored in the freezer at -24°C for further use.

2.3 Sensory evaluation

Sensory evaluation was performed at Matís Reykjavík by a trained group of specialists. The evaluation was performed on all four samples, where four factors were examined for smell and seven factors for taste. The factors for smell were rancidity, fish oil, grass and dulse, but the factors for taste were salt, sour, bitter, fish oil, rancidity, dulse and dried fish. The evaluation was performed twice the same day where 7.5 g of sample were dissolved in 250 mL of water.

2.4 Degree of hydrolysis (OPA)

Preparations

The OPA reagent was prepared by mixing 7.620 g of Borax with 200 mg of SDS in 150 mL of deionized water in a beaker. The solution was stirred and heated to approximately 30°C to get the solution to dissolve completely. Then 160 mg of OPA (97%) were dissolved in 4 mL of 96 % Spiritus fortis Ethanolum. When the reagent was completely dissolved it was added to the solution above among 176 mg of DTT (99%) and mixed together. The solution was then transferred into a 200 mL volumetric flask and the solution was made up to 200 mL with deionized water. The next step was to prepare serine standard by diluting 50 mg of L-Serine in 500 mL of deionized water, in a 500 mL volumetric flask. The sample solutions were prepared by measuring 1 g of sample in 100 mL volumetric flask, and dissolve it in 100 mL of deionized water.

Procedure

To start with three mL of OPA reagent was added to test tubes. First measurement was on 3 mL of deionized water, which was used as a control. Next 400 µl of the serine standard were added to the OPA reagent in the test tubes, and mixed together for 6 seconds in a vortex (Heidolph Reax top, Schwabach, Germany), and then the mixture stood for 2 minutes before being read at 340 nm in the spectrophotometer (Ultrospec 3000 pro Amersham pharmacia biotech, Cambridge, England). When the serine standard had been read, the blanks were prepared from 400 µl deionized water and treated as described above. After the blanks had been read the samples were prepared from 400 µl samples and treated as described above. All samples, standards and blanks were measured in duplicates.

2.5 Rancidity analysis

2.5.1 Peroxide values (PV)

Peroxide value was measured in ten samples, where one samples was treated as a muscle sample and the other nine were treated as homogenous samples. All samples were measured in triplicates.

The muscle sample was treated by weighting 5 g of the sample in a 50 mL centrifuge tube. Then 10 mL of ice cold solvent (Methanol:chloroform (1:1) with 0.25 g of BHT) was added to the tubes, and homogenized (Ultra-turrax, Staufen, Germany) at 6000 rpm for 10 seconds. When it had been homogenized, 5 mL of 0,5M sodium chloride solution was added to the tubes, and mixed together be homogenizing it again. The next step was to centrifuge (TJ-25, Beckman coulter, San Francisco, USA) the solution at 5100 rpm for 5 minutes at 4°C. When the centrifuging had taken place the bottom layer was collected and 500 µl were added to an Eppendorf tube, along with 500 µl of solvent (storage temp) and 5 µl of ammonium thiocyanate and ferrous chloride solution (1:1) and then vortexed. Then the solutions were allowed to sit at room temperature for 10 minutes before 100 µl of the samples were put in a PP microplate (Eppendorf) and measured (Sunrise Tecan, Mannedorf, Switzerland) in duplicates, at 500 nm along with standards.

The homogenous samples were diluted by measuring 0.1 g of sample and 0.9 g of dH_2O, and mixed together in a homogenizer. Then 0.1 g were measured in an Eppendorf tubes along with 600 µl of ice cold solvent, and then homogenized at 6000 rpm for 10 seconds. Then 400 µl of ice cold solvent were added to the Eppendorf tubes along with 250 µl of sodium chloride

solution, and then mixed together by using vortex. The samples were then centrifuged at 5000 rpm for 5 minutes. Thereafter 400 µl of the bottom layer was collected with gel loading tips and put in Eppendorf tubes along with 600 µl of solvent (storage temperature) and 5 µl of ammonium thiocyanate and ferruous chloride solution (1:1). Then the solutions were allowed to sit at room temperature for 10 minutes and measured as described before.

2.5.2 Thiobarbituric acid reactive substances (TBARS)

TBARS were measured in the same ten samples as were measured in PV, where one samples was treated as a muscle sample and the other nine were treated as homogenous samples. All samples were measured in triplicates.

To start with 5 g of muscle sample was weight in 50 mL centrifuge tube along with 5 mL of TCA solution (7.5%), 0.1% Propyl gallate and EDTA and homogenized at 1000 rpm for 1 minute. Then another 5 mL of TCA solution was added to the sample and homogenized again. Thereafter the samples were centrifuged at 5100 rpm for 20 minutes at 4°C. Then 500 µl of the supernatant was collected and put in an Eppendorf tube along with 500 µl of TBA solution (2,883g Thiobarbituric acid). A hole was made on top of each Eppendorf tube with a needle, and then they were placed in a 95°C hot water bath (Julabo 20B, T-275) for 40 minutes, and then the samples were cooled down in ice. When the samples had cooled down, 200 µl of sample was added to microplate in duplicates, and measured in a spectrophotometer at 530 nm along with standards and blank.

The homogenous samples were diluted as described before. Then 0.1 g of diluted sample was measured in an Eppendorf tube along with 600 µl of TCA solution where it was mixed together in a vortex. The samples were then centrifuged at 10.000 rpm for 15 minutes at 4°C, and then 500 µl of the supernatant was collected and put in an Eppendorf tube along with 500 µl of TBA solution and mixed together in a vortex. The rest of the procedure was performed as described above.

2.6 Bioactivity analysis

Bioactivity analysis took place at Matís bioactivity center at Sauðárkrókur, where six different measurements were performed, *in vitro*; ORAC, DPPH, metal chelating, reducing power and ACE inhibitor assay and cellular antioxidant assay.

2.7 Chemical analysis

Chemical analysis was performed at Matís Reykjavík and Matís Neskaupsstaður, where five different analyses were performed on the samples. Salt- (AOAC-Titrino), ash- (ISO, 2002), fat- (AOCS-Ba-3-38, 1998) and water (ISO, 1999) content analysis were carried out at Matís Reykjavík, and protein content analysis (DUMAS) was carried out at Matís Neskaupsstaður.

3 Results and discussion

3.1 Enzyme hydrolysates

Freeze dried powder from fish protein hydrolysates had rather similar weight, but the amount was 40-42 g per sample.

Table 1: Amount of freeze dried powder after hydrolysis with different antioxidants.

Sample	Antioxidant	Amount (g)
Freeze dried FPH-Aa	Ascorbic acid	42
Freeze dried FPH-Se	Seaweed extract	40
Freeze dried FPH-Re	Rosemary extract	40
Freeze dried FPH-C	Control (No antioxidant)	42

3.2 Chemical analysis

Chemical analyses were performed on five different samples.

Table 2: Nutrient content. The table shows the relative (%) content of protein, fat, ash, salt and water in five samples.

Sample	Protein (%)	Fat (%)	Ash (%)	NaCl (%)	Water (%)
Homogenized solution	3.1	0.5	0.8	-	95.7
Freeze dried FPH-Aa	72.1	1.9	18.0	15.6	3.9
Freeze dried FPH-Se	72.6	2.6	19.2	16.9	4.0
Freeze dried FPH-Re	71.5	2.3	18.4	16.9	3.3
Freeze dried FPH-C	74.5	1.5	17.4	15.2	3.6

Table 2 shows that the protein solution is very different from the other samples, where the water content was about 96% compared to the protein powder which was around 4%. The water content in the protein powders is very low in all samples, which is understandable because the samples have been freeze dried, and so the water content should be low.

The protein amount was higher in the powder samples, around 72-75%, then in the solution sample where it was 3%. The reason for that is because the powders have undergone enzyme hydrolysis and thus increasing the protein content by removing existing water.

NaCl content was rather similar in all the samples with value of 16%. Most likely there has been some mistake in the analysis because NaCl content should not be the same in a solution and in a dried powder.

3.3 Degree of hydrolysis

Degree of hydrolysis was measured in four sample solutions that had been hydrolyzed with different antioxidants.

Table 3: Degree of hydrolysis. The table shows the percentage of peptides bond cleaved during the hydrolysis.

Sample	Degree of hydrolysis (%)
Freeze dried FPH-Aa	18.4
Freeze dried FPH-Se	16.0
Freeze dried FPH-Re	18.4
Freeze dried FPH-C	18.1

Table 3 shows that the degree of hydrolysis is rather similar in all of the samples with the average percentage of 17.7%. Hydrolysis with seaweed extract as an antioxidant had the lowest percentage of 16% of peptide bond cleaved.

According to preliminary research, degree of hydrolysis in hydrolyzed fish product is on the range of 15-20% (Nielsen, Petersen and Dambmann, 2001). Therefore the hydrolysates were all successful.

3.4 Peroxide value

Figure 1 shows that the lipid hydroperoxide value decreases from 85 nmol/kg per fat to 1.8 nmol/kg per fat after the mince material had been homogenized.

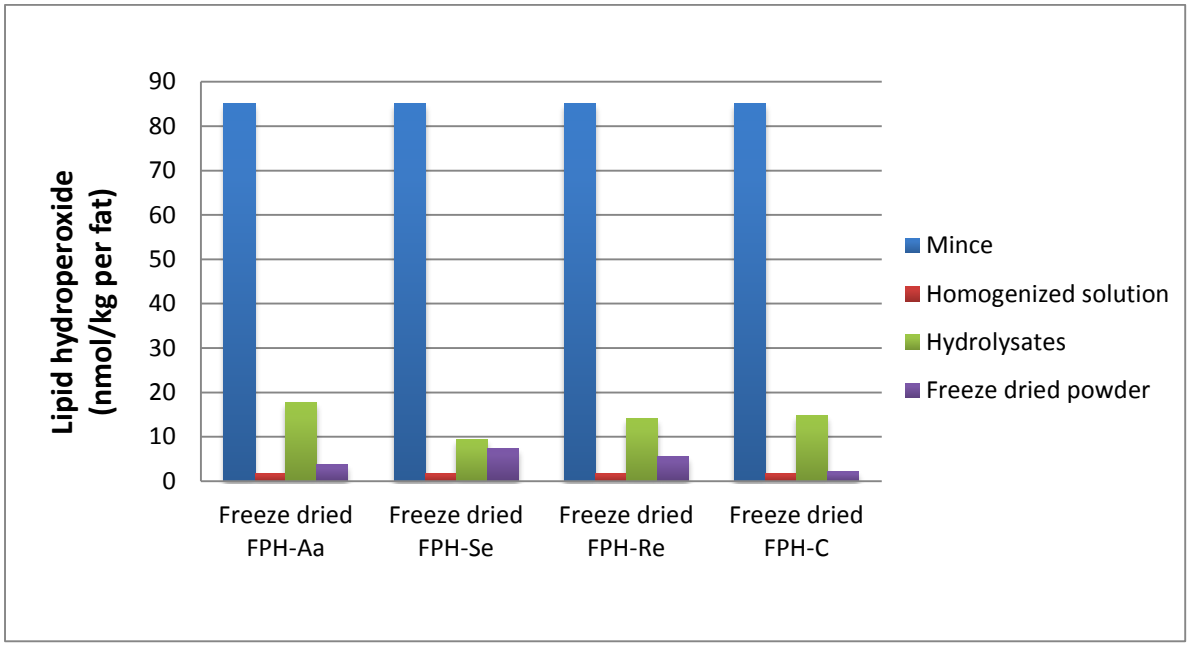

Figure 1: Lipid hydroperoxide value. The figure shows comparison of lipid hydroperoxide (nmol/kg per fat) value of minced Lumpfish head material, homogenized solution of minced lumpfish heads and also value of hydrolyzed solutions and freeze dried powder, with three different antioxidants and a control sample. Results were expressed as mean values standard deviation of triplicate samples.

The lipid hydroperoxide value of the samples measured the lowest in the homogenized solution. Figure 1 shows that lipid hydroperoxide value decreased in all cases after the samples had been freeze dried, because of the peroxy radicals. This indicates that the antioxidants are affecting the rancidity present in the hydrolyzed samples, and therefore the peroxy radicals are changing into secondary products.

The ascorbic acid had the most effect of all the antioxidants, where lipid hydroperoxide value decreased from 17.7 nmol/kg per fat to 3.6 nmol/kg per fat after the hydrolyzed solution had been freeze dried. The seaweed extract showed the least changes of 1.96 nmol/kg per fat between hydrolyzing and freeze drying. The reason for that is because the samples were not all homogenous, and the results show that the control sample was the most homogenous, but the sample with seaweed extract was the least homogenous sample. Therefore the lipid hydroperoxide value measured the highest after freeze drying in the sample hydrolyzed with seaweed extract compared to the other samples.

3.5 TBARS

TBARS value of mince Lumpfish head reduces from 54 nmol MDA/kg per fat to 2 nmol MDA/kg per fat after the material has been homogenized (Figure 2).

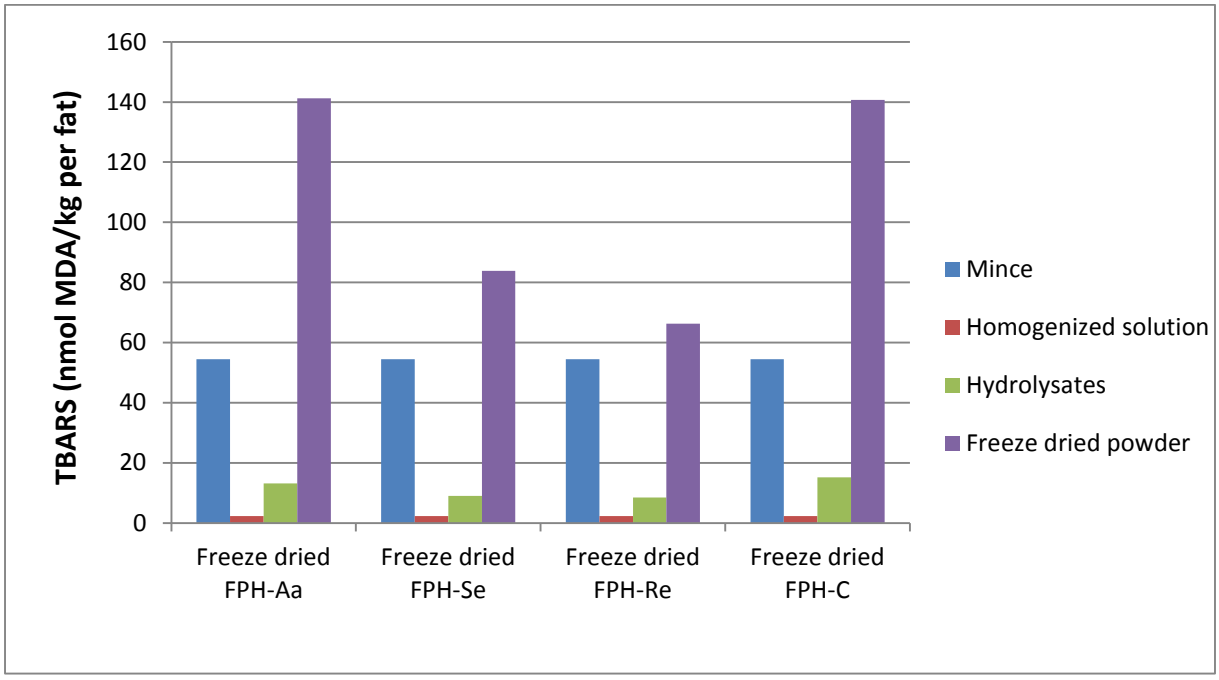

Figure 2: TBARS value. The figure shows comparison of TBARS (nmol MDA/kg per fat) value of minced Lumpfish head material, homogenized solution of minced lumpfish heads and also the value of hydrolyzed solutions and freeze dried powder, with three different antioxidants and a control sample. Results were expressed as mean values standard deviation of triplicate samples.

Figure 2 shows that the TBARS value of the Lumpfish mince measured rather high, but decreased after homogenizing. After hydrolyzing the samples the TBARS value of the samples increased and after freeze drying the value increased even more, from 10-15 nmol MDA/kg per fat in the hydrolyzed solutions to 85-140 nmol MDA/kg per fat in freeze dried powder samples. The reason for the high value of the freeze dried powder samples is most likely due to not enough dilution of the powder samples at the beginning of the measurements. When measuring the absorbance of the samples the freeze dried samples measured over 1.0, which indicates not enough dilution had been performed on the samples. Therefore it would have been necessary to repeat the TBARS measurements of the freeze dried samples in order to obtain significant results.

3.6 Sensory evaluation

The main results of the sensory evaluation can be seen on Figure 3. More detailed report in Icelandic can be found as an Appendix. Results show that the seaweed extract generally contributed to better tasting and smelling FPH with regard to bitter taste, rancidity taste, dried fish taste, rancidity smell, fish oil smell and dulse smell, as compared to the other antioxidants.

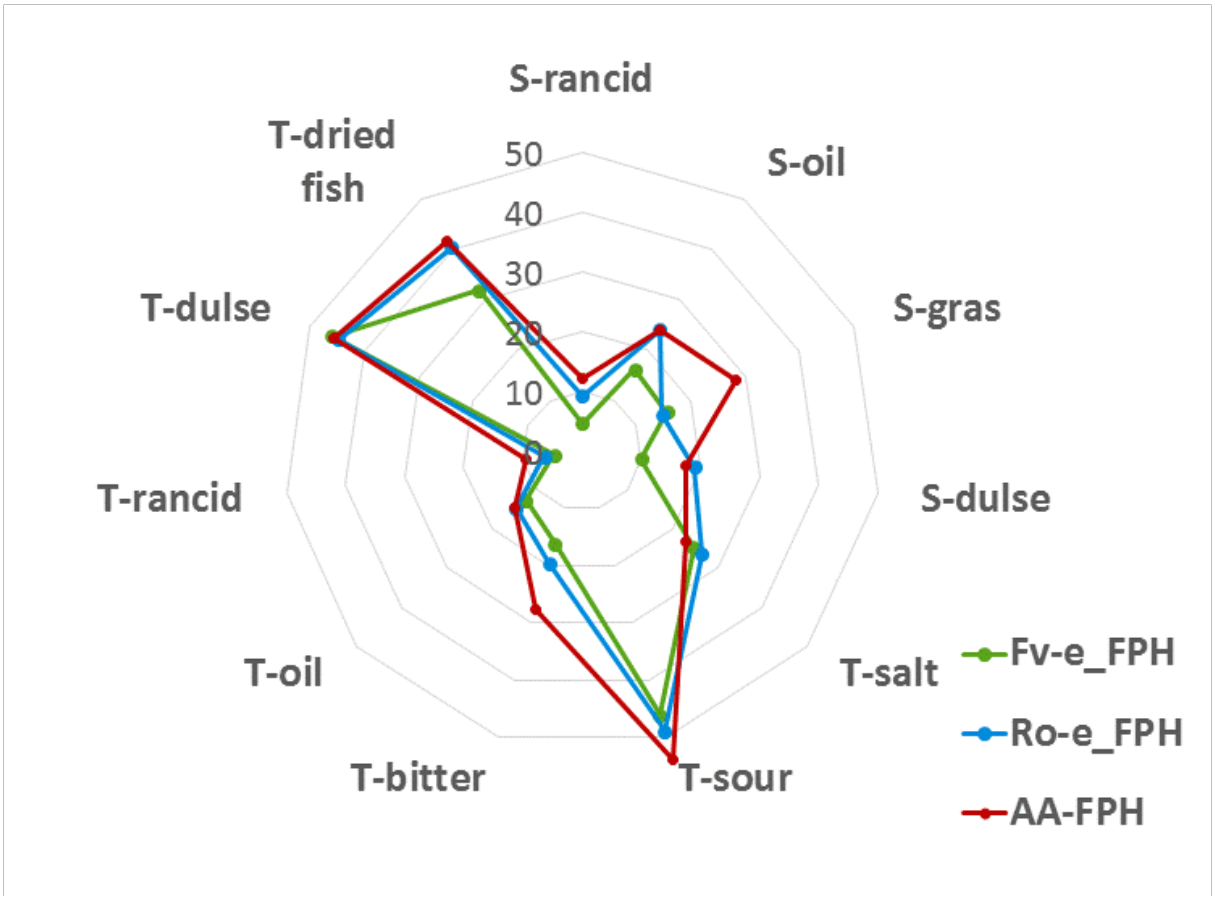

Figure 3: Sensory analysis for the three Fish protein hydrolysates (FPH) samples with antioxidant – Fv-e: Seaweed extract; Ro-e: Rosemary extract; AA: Ascorbic Acid.

3.7 Bioactivity analysis

In general the antioxidant capacity of the hydrolysates was more or the same with the addition of antioxidants (Figure 4 to Figure 6). The rosemary extract gave the overall best results. There are two probable explanations to this: Firstly it inhibited the oxidation during hydrolysis thereby protecting the properties of the hydrolysates themselves, secondly because its own antioxidant properties contributed to increased bioactivity of the final product. Hydrolysates treaded with the seaweed extract did not significantly differ from the control in any of the methods tested.

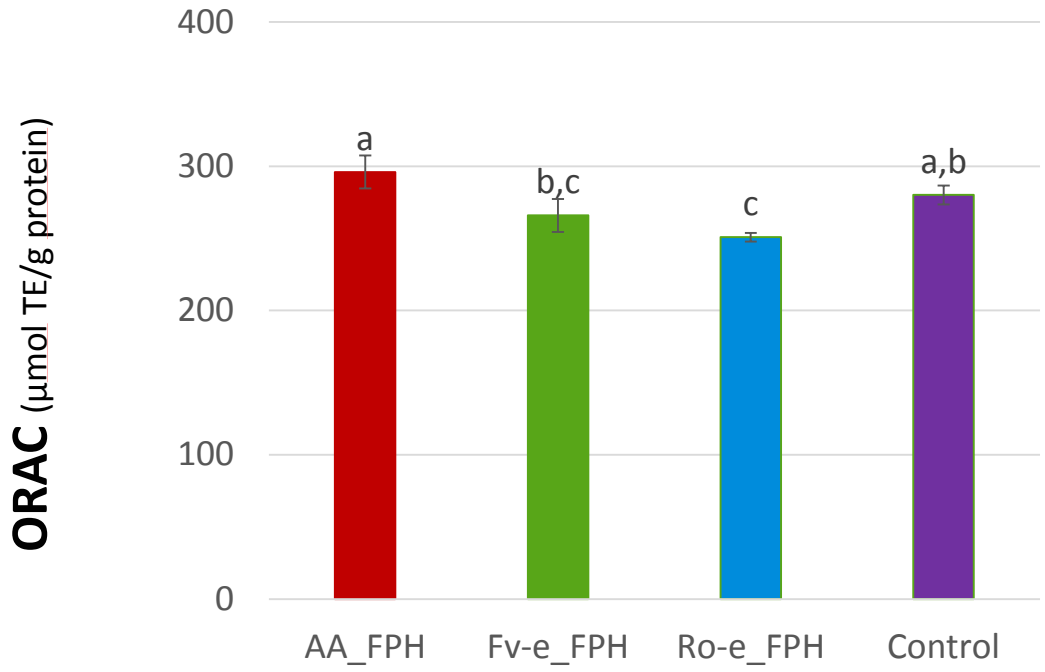

Figure 4: ORAC values for control and the three Fish protein hydrolysates (FPH) samples with antioxidant – Fv-e: Seaweed extract; Ro-e: Rosemary extract; AA: Ascorbic Acid. Average values with standard deviation. Values with the same letter are not significantly different ($p<0.05$).

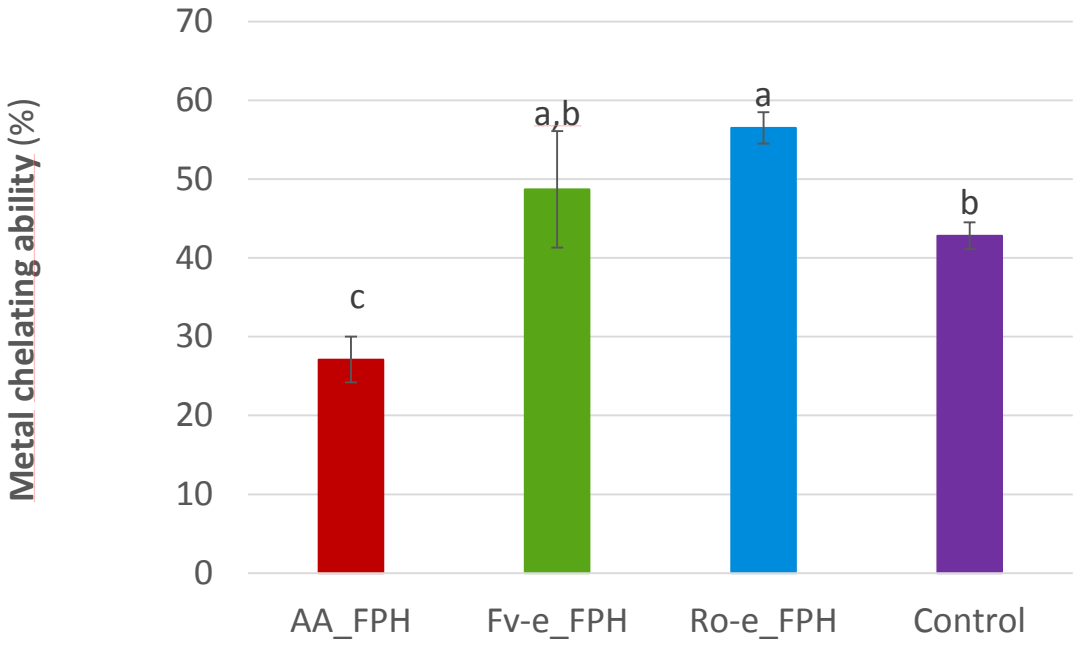

Figure 5: Metal chelating ability for control and the three Fish protein hydrolysates (FPH) samples with antioxidant – Fv-e: Seaweed extract; Ro-e: Rosemary extract; AA: Ascorbic Acid. Average values with standard deviation. Values with the same letter are not significantly different ($p<0.05$).

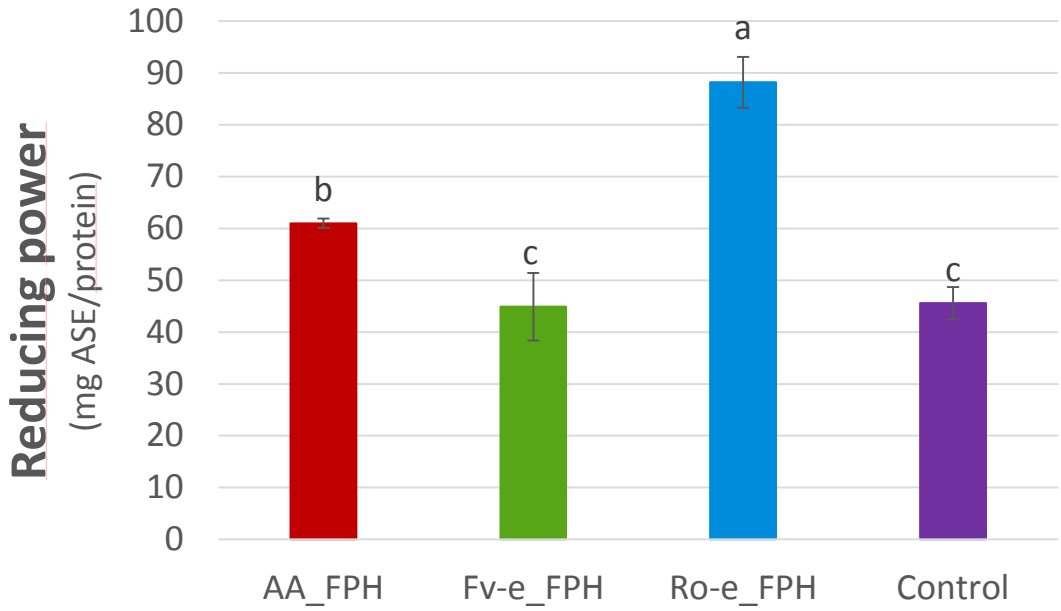

Figure 6: Reducing power for control and the three Fish protein hydrolysates (FPH) samples with antioxidant – Fv-e: Seaweed extract; Ro-e: Rosemary extract; AA: Ascorbic Acid. Average values with standard deviation. Values with the same letter are not significantly different ($p<0.05$).

The samples did not show high ACE inhibition properties – but though higher than the control sample were no activity was detected. The IC_{50} value represents the concentration of hydrolysates needed to inhibit 50% of the ACE. That means that the lower IC_{50} value the stronger is the ability to inhibit ACE. The seaweed extract gave the most promising results. That is in line with previous studies that indicate that *Fucus vesiculosus* extract does possess ACE-inhibiting properties.

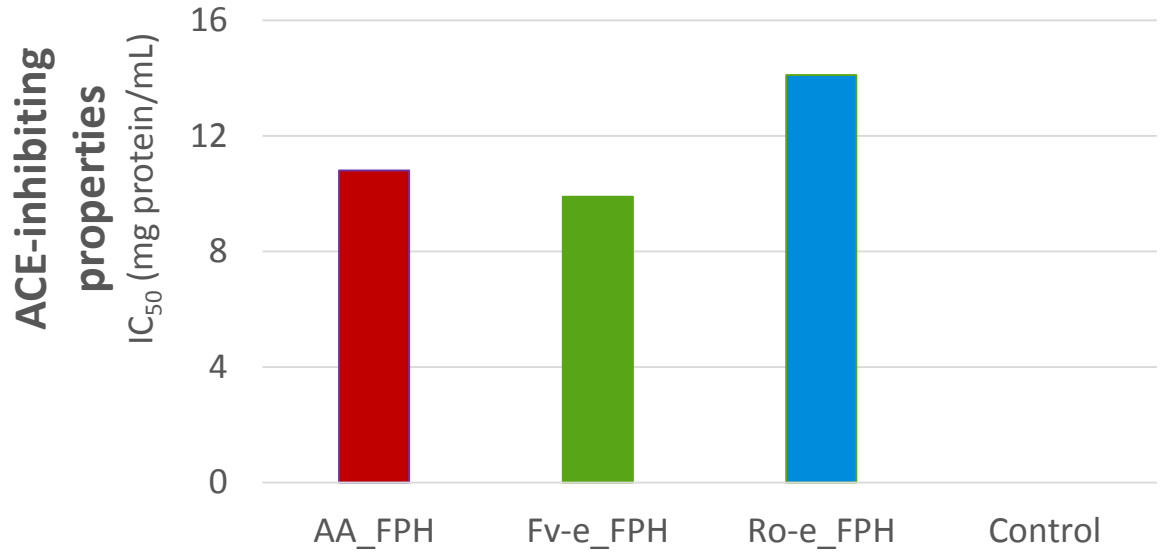

Figure 7: ACE inhibition of the three Fish protein hydrolysates (FPH) samples with antioxidant – Fv-e: Seaweed extract; Ro-e: Rosemary extract; AA: Ascorbic Acid. No activity was detected for the control sample.

4 Conclusion

Samples that had been freeze dried turned out to have similar weight, on the range of 40-42 g.

The chemical analysis results showed that the protein solution had more water content and much lower protein content, compared to the powder samples which had much higher protein content and low water content. NaCl content in the powder samples turned out to be similar to the NaCl content in the solution. Therefore it is considered likely that some mistake had been done in the analysis of NaCl content.

Degree of hydrolysis turned out to be successful in all the samples with the average percentage of 17.7% of peptide bond cleaved during the hydrolysis in the samples.

The lipid hydroperoxide value of the samples measured the lowest in the homogenized solution. The value decreased in all cases after the samples had been freeze dried, which indicates that the antioxidants had an effect on rancidity present in the hydrolyzed samples. The samples turned out to be not all homogenous, resulting in different lipid hydroperoxide value in control sample compared to samples with antioxidants.

The TBARS value measured high in the Lumpfish mince but decreased after homogenizing. After hydrolyzing the samples the TBARS value of the samples increased and after freeze drying the value increased even more. The reason for this rather high value of the freeze dried samples is considered to be due to not enough dilution of the samples at the beginning of the measurements. Therefore it would have been necessary to repeat the TBARS measurements of the freeze dried samples

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APPENDIX

A. Framkvæmd

Skynmat var framkvæmt á fjórum tilraunahópum af fiskipróteinum í mars 2014. Munur milli tilraunahópa fólst í mismunandi þráavarnarefnum sem bætt var út í próteinin. Lýsingar hópa eru sýndar í töflu 1 en í umfjöllun verða þeir kallaðir A, B, C og D. Sýnin voru metin eftir myndrænu prófi, (GDA - Generic Descriptive Analysis), þar sem skilgreindir matsþættir voru metnir til að lýsa einkennum í lykt og bragði af þjálfuðum skynmatshópi (Lawless and Heymann, 2010). Sjö dómarar sem allir höfðu reynslu af skynmati (ISO, 1993) og þekktu vel aðferðina tóku þátt í skynmatinu. Matsþættir voru skilgreindir í einum þjálfunartíma en þeir voru 11 talsins og eru sýndir ásamt skilgreiningum í töflu 2. Hver matsþáttur var metinn eftir styrk á ókvarðaðri línu sem í úrvinnslu var kvörðuð frá 0-100. Fiskipróteinin voru leyst upp í vatni í styrknum 3g/100ml og leystust próteinin vel upp. Hvert sýni var um 10ml af lausn borin fram í glæru plaststaupi. Öll sýni voru dulkóðuð með þriggja stafa númeri og borin fram í mismunandi röð til að takmarka áhrif sýnaraðar á niðurstöður. Skynmatsforritið Panelcheck (V1.4.0) var notað til að skoða frammistöðu dómara. Forritið NCSS 2000 (NCSS, Utah, USA) var notað til að skoða mun milli hópa en til þess var notað ANOVA (glm) og Duncan´s test. Leiðrétt var fyrir notkun dómara á skala. Í úrvinnslu var miðað við 95% öryggismörk og munur því talinn marktækur ef $p < 0,05$.

Tafla 1. Lýsingar tilraunahópa.

hópur	þráavarnarefni
A	Ascorbic acid
B	Seaweed extract
C	Rosemary extract
D	Viðmið - ekkert þráavarnarefni

Tafla 2. Skynmatsþættir fyrir fiskiprótein og skilgreiningar á þeim. L-lykt, B-bragð.

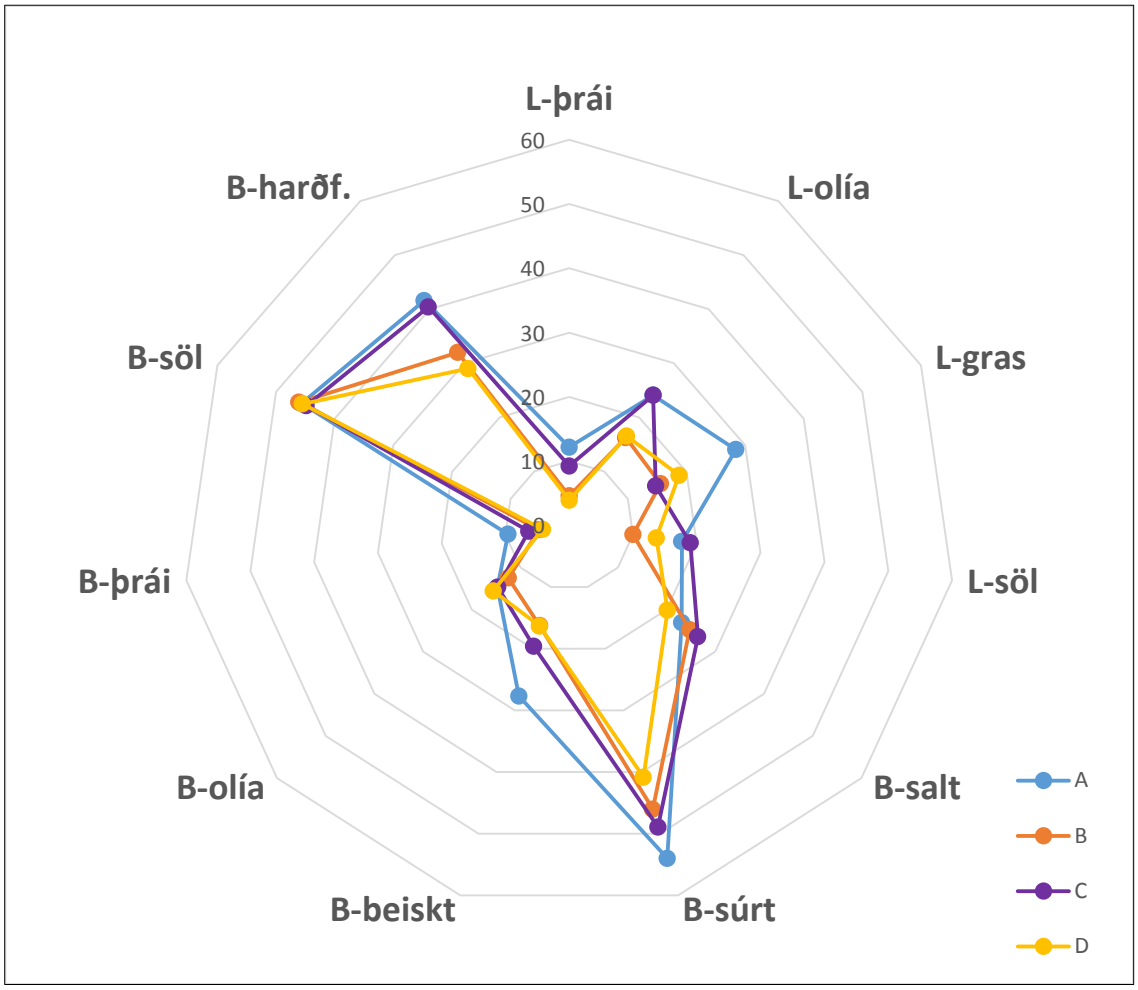
	skynmatsþáttur	stytting	skali	skilgreining
LYKT				
	þrái	L-þrái	engin || mikil	þráalykt
	fiskolía	L-olía	engin || mikil	fiskolía, lýsi, loðna
	gras	L-gras	engin || mikil	gras, hey, súrhey
	söl	L-söl	engin || mikil	söl, sjávarlykt
BRAGÐ				
	salt	B-salt	ekkert || mikið	salt bragð
	súrt	B-súrt	ekkert || mikið	súrt bragð
	beiskt	B-beiskt	ekkert || mikið	beiskt bragð
	fiskolía	B-olía	ekkert || mikið	fiskolía, lýsi, loðna
	þrái	B-þrái	ekkert || mikið	þrái
	söl	B-söl	ekkert || mikið	söl
	harðfiskur	B-harðf.	ekkert || mikið	harðfiskur, eftirbragð, TMA

B. Niðurstöður

Nokkur munur var milli hópa í lykt og bragði. Meðaltöl skynmatsþátta eru sýnd í töflu 3 og í stjörnuriti á mynd 1. Hópur A og C höfðu meiri þráalykt en hópar B og D. Hópur A hafði meiri fiskolíulykt og súrt bragð en hópur D og meiri graslykt, beiskt bragð og þráabragð en aðrir hópar. Hópur B hafði minni sölvalykt en hópar A og C og hópur D hafði minna harðfiskbragð en hópar A og C. Ekki var munur í söltu bragði, sölvabragði eða fiskolíubragði milli hópa. Almennt höfðu allir tilraunahópar vart greinanlegan þráa en greinilega lykt af fiskolíu, grasi og sölvum. Bragð einkenndist af mjög súru bragði, bragði af sölvum og harðfiski. Einnig var nokkuð beiskt og salt bragð af öllum hópum, greinilegur vottur af fiskolíubragði og vart greinanlegt þráabragð. Almennt voru hópar B og D frekar líkir og var hvergi munur milli þessara hópa. Hópur A var lyktar og bragðmeiri en aðrir hópar og einkenndist aðallega af graslykt, og beisku bragði.

Tafla 3. Meðaltöl skynmatsþátta fyrir hópa A, B, C og D og p-gildi fyrir mun milli hópa. Mismunandi bókstafir innan línu gefa til kynna marktækan mun milli viðkomandi hópa.

skynmatsþáttur	A	B	C	D	p-gildi
LYKT					
þrái	12 **a**	5 **b**	9 **a**	4 **b**	0,000
fiskolía	24 **a**	16	24	17 **b**	0,050
gras	28 **a**	16 **b**	15 **b**	19 **b**	0,004
söl	18 **a**	10 **b**	19 **a**	14	0,011
BRAGÐ					
salt	23	25	26	20	0,239
súrt	54 **a**	46	49	41 **b**	0,031
beiskt	28 **a**	16 **b**	20 **b**	16 **b**	0,000
fiskolía	15	13	15	16	0,881
þrái	10 **a**	5 **b**	6 **b**	4 **b**	0,000
söl	46	46	45	46	0,997
harðfiskur	42 **a**	32	40 **a**	29 **b**	0,020



Mynd 1. Meðaltöl skynmatsþátta fyrir hópa A, B, C og D.

C. Ályktanir

Mjög lítill þrái fannst af viðmiðunarsýni og því var erfitt að sjá áhrif þráavarnarefna á þráamyndun í próteinunum en þráabragð var metið mest í hópi A. Harðfiskbragð, sem einnig getur gefið til kynna oxun, var mest í hópum A og C en minnst í viðmiðunarhópi. Almennt var hópur B frekar líkur viðmiðunarhópi og hvergi var munur milli þessara tveggja hópa. Hópur A var almennt lyktar og bragðmeiri en aðrir hópar og einkenndist aðallega af graslykt, og beisku bragði.

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VIÐAUKI III

Bioactive Properties and Chromatographic Profile of Lumpfish Hydrolysate

Siegfried Reash
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Margrét Geirsdóttir

Contents

Abstract

This thesis describes the bioactivity and chromatographic profile of Lumpfish (*Cyclopterus lumpus*) hydrolysate produced via enzymatic hydrolysis. Antioxidant and antihypertensive properties of various fish hydrolysates have been proven in previous experiments. In particular the impact of various proteases and different times of hydrolysis on the bioactive properties of the hydrolysate are the main focus in this study. The commercial enzymes used are: Protease M, Protease P and Protamex, hydrolysing Lumpfish fillets for 1h, 3h and 12h. The aim of this project is to find a time-saving and cost-reducing workmanship to produce highly bioactive fish hydrolysates. Therefore the raw material was minced, pH and temperature adjusted, controlled hydrolysed and purified via centrifugation and ultrafiltration. The freeze dried hydrolysates were characterized with reversed-phase HPLC and size exclusion chromatography. Data about potential antioxidant and antihypertensive properties were compiled via different bioactive assay including: Metal chelating activity, DPPH radical scavenging capacity, oxygen radical absorbance capacity (ORAC), cellular antioxidant activity (CAA) and angiotensin-converting enzyme (ACE) inhibitory activity. It can be detected that all tested variants of Lumpfish hydrolysate reveal bioactive properties. Samples hydrolysed with Protease M and Protease P showed in comparison to Protamex a higher bioactivity in all performed assays. A hydrolysis time of 1h supported DPPH scavenging abilities, whereas 3h and 12h hydrolyses were more suitable to achieve high ORAC, CAA and ACE inhibitory activities on the Lumpfish hydrolysate.

Showing once more remarkable bioactivity of hydrolysed marine organisms, it can be encouraged to do further research on new species and to optimize the way of producing marketable and healthy fish hydrolysates.

1 Introduction

1.1 Fish Proteins

Fish muscle tissue consists of three different protein types. The *structural proteins* insist of actin, myosin, tropomyosin and actomyosin, which constitute 70-80% of the total protein content (40% in mammals). They make up the contractile apparatus of the fish, which is responsible for the muscle movement. The rest is completed by 20-30% of sarcoplasmic (myoalbumin, globulin, enzymes) and 3-10% of connective tissue proteins (collagen). The amino acid composition from corresponding proteins is in comparison to mammalian muscles similar with slight differences in physical properties. For the distinction between fishes, the unique sarcoplasmic protein band pattern of each fish species is used, obtained via the isoelectric focusing method. The connective tissue with the different types of collagen (found in skin, swim bladder and the myocommata in muscle) is the trigger for the swimming behaviour of the fish species and is in structure similar to mammals, but shows fewer and more labile cross-links than collagen from warm-blooded vertebrates (Ababouch, 2008)

1.2 Peptides

Peptides are linear polymers joined together with amino acids forming a protein with at least around 50 amino acids in the polypeptide chain. In molecular mass the range of peptide varies between 130 – 5,500 g mol^{-1} (Da), dependant on the amino acid composition. The linkage between one and another amino acid is made on the α-carboxyl group and the α-amino group and is named peptide bond. The formation of a dipeptide (2 amino acids linked together) via the peptide bond is synthesized through the loss of a water molecule or vice versa by hydrolysis (Fig.1.1).

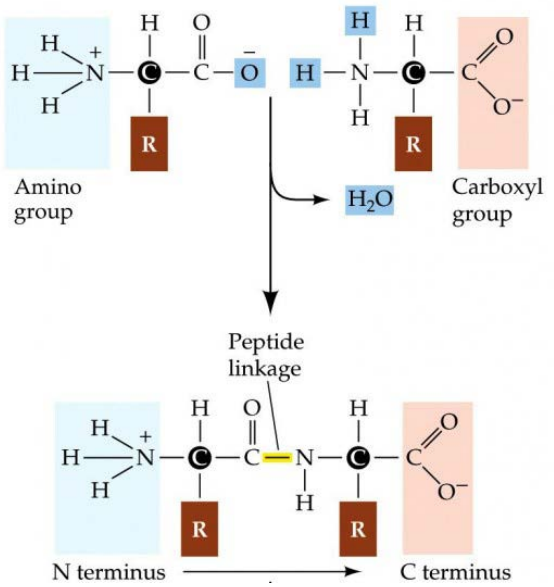

Fig.1.1: Shown is the peptide-bond formation as an example for amino acid synthesis (©2001 Sinauer Associates, Inc.).

The sequence of a polypeptide chain is written beginning at the amino end up to the carboxyl-terminal residue. This chain of amino acids forms a repeating part rich in potential hydrogen binding carbonyl groups (C=O), called *backbone*, and a variable part constituted of amino acid side chains. The linear polypeptide chain can sometimes be cross-linked over disulphide bonds with cysteine. Geometry reveals a planar backbone with 6 atoms (C_α,C, O, N, H and C_α) lying on one plane. A double bond character within CO and NH (switching between single and double bond) enables a stable and constrained backbone without rotations. There are only 2 configurations possible for a peptide bond, namely when the α-carbons are on opposite sides (*trans*-) or in the other case the2 α-carbons are on the same side (*cis*-configuration), which is the rarer configuration.

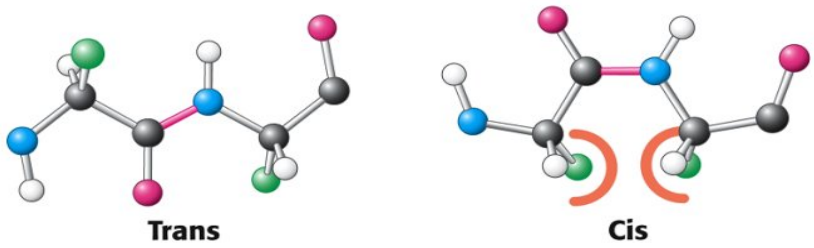

Fig.1.1: Illustrated are the cis- and trans-configurations of the peptide bond. (http://www.imb-jena.de/Bioinformatics)

Apart from the peptide bond free rotations are possible (between amino group and α-carbon, and between α-carbon and carbonyl group) which allows the protein to fold in many different ways and exert specific functions (Berg, Tymoczko & Stryer, 2012).

1.3 Bioactive peptides

Specific protein fragments with a positive impact on body functions and ultimately on health are defined as bioactive peptides. In cell physiological terms they act like hormones with specific interactions to certain cell receptors thereby triggering physiological response and metabolic regulation (Sharma, Singh, & Rana, 2012). The diverse functionality of bioactive peptides on humans physiology are described in many studies and include antihypertensive, antioxidant, anticancer, antimicrobial, and opioid activities as well as immunomodulatory and cholesterol-lowering effects. Bioactive peptides are extrinsically brought to the gastrointestinal tract from various food sources (Shahidi & Zhong, 2008). The so called “food-derived bioactive peptides” can be present genuine in food or generated in vivo (intestinal) or in vitro (food processing) via hydrolysis (Hartmann & Meisel, 2007). Numerous animal and plant proteins encrypt bioactive peptides (Tab.1.1), which become only active if released from the parent protein (Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011). The bioactivity of these peptides is primarily dependent on size (usually 2-20 amino acids), amino acid composition and sequence (Najafian & Babji, 2011); (Jeon, Byun, & Kim, 1999)

1.3.1 Antihypertensive peptides from fish

The main antihypertensive peptides are inhibitors of the Angiotensin converting enzyme, which plays an important role in the renin – angiotensin system (RAS) (Vercruysse, Van Camp, & Smagghe, 2005). RAS is triggered when granular cells of the juxtaglomerular apparatus in the kidney release the hormone renin acting as an enzyme on angiotensinogen. The catalysis converts angiotensinogen to angiotensin I, which is now available for the angiotensin converting enzyme (ACE) to form angiotensin II. From now on the regulation of blood pressure is intact,

whereby angiotensin II stabilizes systemic blood pressure via activating smooth muscle of arterioles as a vasoconstrictor and stimulator of sodium reabsorption.

Tab.1.1: Examples of bioactive peptides encrypted in proteins form various food sources with a specific physiological effect (Ryan et al., 2011).

Food source	Encrypting protein	Peptide (sequence)	Effect
Soy	Soy protein	NWGPLV	antihypertensive
Fish	Fish muscle protein	LKP, IKP, LRP	antihypertensive
Meat	Meat muscle protein	IKW, LKP	antihypertensive
Broccoli	Plant protein	YPK	antihypertensive
Egg	Ovotransferrin	OTAP-92	antimicrobial
Rice	Rice albumin	Oryzatensin	Immunomodulatory
Wheat	Wheat germ protein	Peptides not specified	antioxidant
Milk	Lactoferrin	Lactoferricin	antimicrobial

Both properties and the indirect release of aldosterone from the adrenal cortex make angiotensin II a potential hypertensive hormone (Marieb & Oehn, 2010). In pathological conditions, there is an overdose of Angiotensin II available resulting in severe hypertension. Till date, many discoveries have demonstrated that bioactive peptides from food source can competitively inhibit to a certain extent ACE and therefore lowering blood pressure (Udenigwe & Aluko, 2012). Some of this food-derives bioactive peptides come from fish.

The first ACE inhibitory peptides from fish source were found in sardines around 20 years ago. After some more identifications of antihypertensive peptides from shellfish, tuna and salmon, they were classified to be rather small chained (200-600Da), polar and containing few hydrophobic amino acids in their sequence (Ryan et al., 2011). An isolated peptide (amino acid chain LKPNM) from dried bonito showed in studies an IC_{50} (inhibitory concentration) value of 2.4 µM, which means

that this peptide inhibit 50% of ACE with an application concentration of 2.4µM (Fujita & Yoshikawa, 1999). Another study proved ACE inhibitory potential of tripeptides (amino acid chain GPL) isolated from Alaskan pollack skin gelatine hydrolysate (Byun & Kim, 2002). An experiment to gain more value out of fish processing waste approved that low molecular weight fractions (<10kDa) of tilapia hydrolysates exhibit a higher ACE inhibitory activity than unfiltered fractions (Raghavan & Kristinsson, 2009). Bioactive peptides derived from fish can exert antihypertensive and antioxidant effect at the same time. This was shown in a recent study on Pacific hake fish protein hydrolysates (Samaranayaka, Kitts, & Li-Chan, 2010). These promising facts on fish-derived bioactive peptides motivate research groups all over the world to expand the knowledge on the diverse health aspect of fish with further studies.

1.3.2 Antioxidant peptides from fish

The nature of antioxidant peptides is to scavenge and quench reactive oxygen/nitrogen species (ROS/RNS) and inhibition of ROS-induced oxidative damage on biological macromolecules such as lipids, proteins and DNA. In addition, some of them are able to enhance the activities of antioxidant enzymes or inhibit the expression of free radical generating enzymes (Udenigwe & Aluko, 2012). These free radicals carrying reactive species are in an unstable configuration and release their energy in reactions with adjacent molecules (Rahman, 2007). ROS, with the most common free radicals superoxide anion ($O_2^{-\bullet}$), perhydroxyl radical ($HO_2^{\bullet}$), hydroxyl radical (-OH) and hydrogen peroxide (H_2O_2), are products from cell metabolism and play an important role in cell signalling, gene expression, ion transportation and apoptosis (Fig.1.3). If speaking about oxidative stress, the hydroxyl radical is the major attacker for bases in nucleic acids, double bonds in unsaturated fatty acids and amino side chains in proteins. Cells use as self-defence against ROS damage intracellular enzymes (superoxide dismutase, catalase, glutathione peroxidise), metal chelating and free radical scavenging substances (vitamin E, C). Nevertheless, the own body defence is sometimes deficient in strength against free radicals when cell dysfunction and environmental stress raise the level of ROS to a dramatic extent, which can significantly contribute to cancer,

Alzheimer's disease and rapid aging. In order to reduce the cellular oxidative stress, additional uptake of food with antioxidants would help to maintain health (Lü et al., 2010).

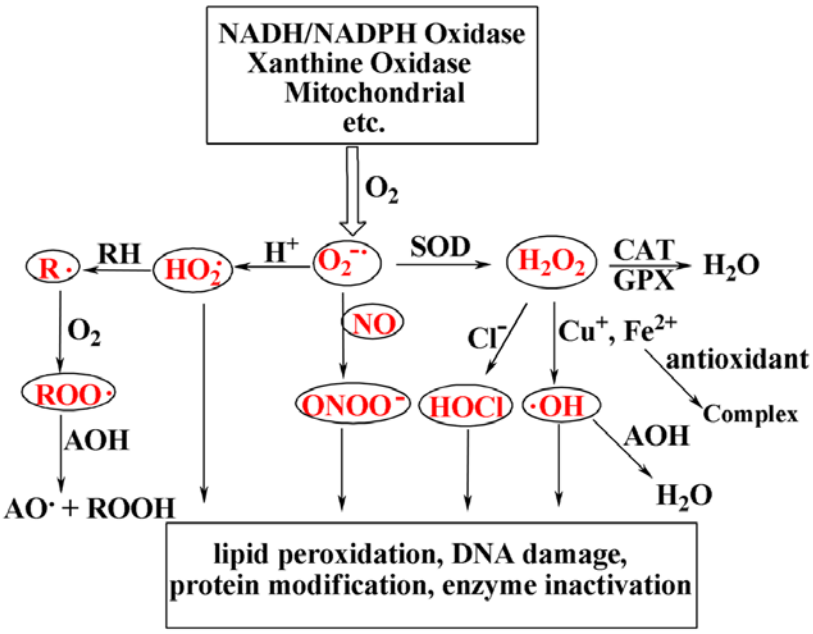

Fig.1.2: Schematic drawing of the generation and reaction of free radicals in the cell. $O_2^{-\bullet}$ (superoxide anion), H_2O_2 (hydrogen peroxide), -OH (hydroxyl radical), HOCl (hypoclorous acid), NO (nitric oxide), $ONOO^-$ (peroxynitrite), $HO_2^\bullet$ (perhydroxyl radical), R• (lipid alkyl radical), ROO• (lipid peroxyl radical), RH (lipid), ROOH (lipid hydroperoxide), CAT (catalase), GPX (glutathione peroxidise) and SOD (superoxide dismutase) (Lü et al., 2010).

Such antioxidants were also discovered in enzymatically hydrolysed fish muscles of different marine species (Najafian & Babji, 2011). Derived peptides from flounder fish hydrolysis using α-chymotrypsin as protease showed a high antioxidant activity among other tested enzymes (Ko, Lee, Samarakoon, Kim, & Jeon, 2013). Hoki skin gelatine hydrolysates obtained a significant scavenging activity on DPPH, carbon-centered and superoxide radicals when hydrolysed with trypsin (Mendis, Rajapakse, & Kim, 2005). A well purified and characterized peptide (amino acid chain KTFCGRH) from croaker could achieve successful in vivo results by increasing the activity of endogenous cellular antioxidant enzymes (e.g. catalase) in Wistar rats (Nazeer, Kumar, & Jai Ganesh, 2012). Not only did hydrolysates from fish muscle proteins and skin contain antioxidant peptides demonstrate a study about cod backbone hydrolysates (Šližytė et al., 2009). In cellular based assays an antioxidant activity was found with Threadfin bream surimi byproducts, where hydrolysates from

frame, skin and bone protected HepG2 cells against *tert*-butyl hydroperoxide-induced oxidative damage (Wiriyaphan, Chitsomboon, & Yongsawadigul, 2012). Along with proven antihypertensive effects represent antioxidant studies on fish protein-derived bioactive peptides the potential health benefits of fish hydrolysates. Their intake is discussed to be in forms of pharmaceuticals, nutraceuticals or as a functional food ingredient (Najafian & Babji, 2011).

1.4 Enzymatic hydrolysis of fish muscle proteins

Three techniques have been established so far to produce bioactive peptides and are partly applied in fish processing. These methods comprise: solvent extraction, microbial fermentation and enzymatic fermentation of food proteins. Solvent extraction is mainly used in laboratory scale and displays impractical drawbacks like low selectivity, solvent residue and weak extraction efficacy. The gain of bioactive peptides via microbial fermentation has more application in meat and specially milk processing. The method of choice for fish protein-derived bioactive peptide production is the enzymatic hydrolysis, which is considered not to generate toxic by-products or leave harmful residues, but rather results in products of high functionality and good organoleptic properties (Najafian & Babji, 2011). Production of fish peptides via enzymatic hydrolysis starts with sarcoplasmic and myofibrillar protein extraction by adding water and adjusting the pH-value and temperature to preferable conditions for enzymes (Thorkelsson & Kristinsson, 2009). Only a well homogenized and adjusted fish mince is ready for the enzyme addition, which are normally dosed to achieve 0.5-2% of the total protein content. The main physiocochemical conditions of the reaction media are summed up as followed:

- time
- temperature
- pH-value
- enzyme/substrate (E/S) ratio

During hydrolysis, the environmental conditions are kept stable and the process is running until a certain degree of hydrolysis (DH) is reached. DH is defined as the ratio of cleaved peptide bonds to the total protein content in the hydrolysate. The

termination of the hydrolysis is mostly done via heat treatment, which may adversely influence the functionality of the bioactive peptides (Kristinsson, 2005). As indicated before, the degree of hydrolysis is the main benchmark to check, compare and conclude the final product on its posterior tested bioactivity. How crucial DH for bioactive properties can be trailed in some studies (Geirsdottir et al., 2011); (Klompong, Benjakul, Kantachote, & Shahidi, 2007), where a higher DH resulted in a stronger bioactivity. There are many established methods to determine the DH based on different principles, which constitute sometimes an obstacle by comparison of results between studies. One can measure the released protons during hydrolysis as a parameter, called pH stat method. The trichloroacetic acid soluble nitrogen (SN-TCA) method determines the free TCA-soluble nitrogen in the solution. Three more methods, trinitrobenzenesulfonic acid (TNBS), formol titration and o-phthaldialdehyde (OPA), are measuring the generated amino groups during hydrolysis (Rutherfurd, 2010). OPA is the newest invention with considerable advantages such as time saving, high accuracy and less toxicity, and therefore selected for this thesis (Nielsen, Petersen & Dambmann 2001). O-phthaldialdehyde is a strong derivatizing agent for amino groups in the presence of reduced sulfhydryl groups supplied by the addition of various reagents like dithiothreitol (DTT) or β-mercaptoethanol (Fig.1.4). The product of this reaction is a fluorescent moiety, which can be detected with a spectrophotometer at 340nm. The fact that OPA does not really react with proline nor cysteine and shows a low derivatizing potential to insoluble proteins and peptides leads to an slight underestimation of the DH, which should be kept in mind for comparison with other methods (Held, 2006); (Rutherfurd, 2010).

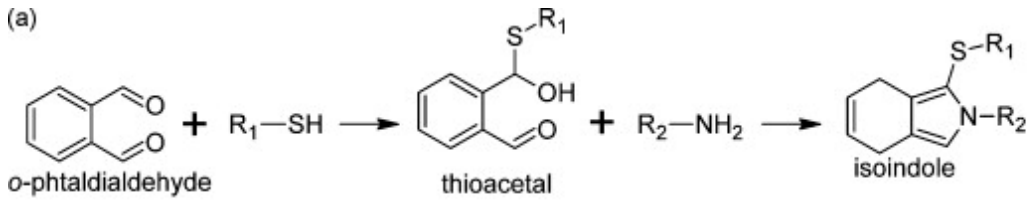

Fig.1.3: The reaction mechanism of OPA with an amino group forming the fluorescent isoindole complex with a thioacetal as an intermediate (Kyprianou et al., 2010).

1.5 Proteases

A protease is an enzyme, which cleaves peptides bonds with many different mechanisms. The process is generally called proteolysis and the enzymes are classified as hydrolases, because of the need of one water molecule during the reaction. They can be discerned into 5 major catalytic classes (serine, threonine, cysteine, aspartic acid, metalloprotease and glutamic acid protease) and 63 different families. Proteases can also be classified according their mode of attack on proteins into 2 classes: Endo- and Exoproteases. Endoproteases break down proteins from the interior and usually leave bigger peptides with more amino acids. Exoproteases slowly cleave amino acids from both ends of the protein and thus producing single amino acids (Puente et al., 2003); (Berg, Tymoczko & Stryer, 2012). A lot of proteases with different functions are present in the human body, for example elastase, cathepsin G in leukocytes; trypsin and chymotrypsin for food digestion in the duodenum; or pepsin in the stomach. The proteases applied in industry are often blends of endo- and exoproteases representing a huge, nearly inscrutable offer. This leads to a high diversity of tests being performed with different proteases to detect an optimal way for producing fish hydrolysates with an a high bioactivity (Thorkelsson & Kristinsson, 2009)

1.6 Chromatography – Peptide purification

Due to the complexity of peptide, especially in hydrolysates, it is not possible to use the rather simple crystallization method as purification strategy, which applies to most of the organic compounds. Nowadays the solution to purify a high complex peptide-protein mixture lies in the use of combined multidimensional liquid chromatography. The general principle of chromatographic purification is the interaction of the peptide with a stationary phase carried in a mobile phase. Reversed-phase, size exclusion (used for this thesis) and ion exchange chromatography are documented as the most efficient and industrially applied chromatographic techniques for peptide purification (Andersson, Persson, & Laboratories, n.d.); (Sandra et al., 2009); (Conrads, Janini, Veenstra, & Nci-frederick, 2002).

1.6.1 Reversed-phase Chromatography

Contrary to NP, RP uses a nonpolar bonded coating as the stationary phase. The mechanism of separation is referred to hydrophobic interactions between the peptide and the immobilized ligand (stationary phase). Nonpolar, hydrophobic peptides are eluting later than polar, hydrophilic ones, because of the stronger adsorption to the nonpolar ligand. The R-group of the matrix is coated with alkyl chains e.g., C4, C8, C18 constituting the nonpolar stationary phase. The carbon groups are aligned perpendicular to the silica surface giving a broom-like structure. Usually a polar liquid is functioning as elution buffer in the mobile phase. This is predominantly a mixture of water, methanol or acetonitrile, sometimes in combination with a low concentration of acid to keep the pH below 7.5, because silica gel matrices (not valid for polystyrene matrices) can easily dissolve above this pH-value (Skoog, Holler & Nieman, 1997); (http://www.infoagil.ch); (Amersham Bioscience, 18-1134-16).

1.6.2 Size exclusion Chromatography

The mode of operation of size exclusion chromatography is the separation of peptides according to their differences in size. The mobile phase can be either non-aqueous, Gel permeation chromatography (GPC), or aqueous, Gel filtration chromatography (GFC), passed through a column filled with a porous gel bed. Unlike IEX, the molecules don't bind to the stationary phase, but diffuse into the beads if they are small enough to pass through the spherical particle holes. The smaller sized molecules can go easier into the beads leading to a longer retention time in the stationary matrix. Whereas the bigger molecules have problems to enter the beads or even can't pass through the holes revealing a faster flow through and the first peaks in the chromatogram. This separation technique just needs one buffer, which composition can be beneficially adjusted to suit the type of sample, or the requirements for further purification, analysis or storage. By contrast the stationary phase, normally made of styrene-divinylbenzene copolymers or hydroxylated silica, has to withstand high temperatures and pressures as well as a broad pH range (Amersham Bioscience, 18-1022-18).

2 Materials and methods

2.1 Chemicals

All chemicals were of analytical grade and the water used was distilled and sterile filtered through a Millipore device. The chemicals and solutions used during the whole thesis are listed in the table 2.1 below.

Tab 2.1: Shows the list of all applied chemicals in the trials with the stated name of the manufacturer.

Chemical	Source
2,2-diphenyl-1-picryhydrazyl (DPPH)	Merck
2',7'-dichlorofluorescein diacetate (carboxy-H2DCFDA)	Sigma-Aldrich
3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate (ferrozine)	Sigma-Aldrich
Sodium phosphate dibasic	Sigma-Aldrich
Acetonitrile	Sigma-Aldrich
Ammonium chloride	Merck
Ammonium persulfate (APS)	Sigma-Aldrich
Ammonium sulphate	Merck
Ammoniumhydrophosphate	Merck
Angiotensin-converting enzyme (ACE) from rabbit lung	Sigma-Aldrich
Bromophenol blue	Sigma-Aldrich
Coomassie brilliant blue	Merck
β-mercaptoethanol	Sigma-Aldrich
Dipotassium hydrogen orthophosphate	Riedel-De Haën
Disodium tetraborate decahydrate (Borax)	Merck
Dithiothreitol (DTT)	Sigma-Aldrich
Ethanol	Gamla apotekid
Ethylendiaminetetraacetic acid (EDTA)	Merck
Ferrous chloride	Sigma-Aldrich

Fetal bovine serum (FBS)	GIBCO
Fluorescein sodium salt	Sigma-Aldrich
2,2'-Azobis-2-methyl-propanimidamide, dihydrochloride (AAPH)	Cayman Chemical
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)	Cayman Chemical
Glycerol	Sigma-Aldrich
Hanks' Balanced Salt Solution (HBSS)	GIBCO
Hexan	Sigma-Aldrich
Hydrochloric acid	Sigma-Aldrich
Isopropanol	Sigma-Alrdich
Methanol	Riedel-De Haën
Minimum Essential Medium α (MEMα)	GIBCO
o-aminobenzoylglycine (Abz-Gly)	Bachem
o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe(NO2)-Pro)	Bachem
Ortho-phthaldialdehyde (OPA)	Sigma-Aldrich
Phosphate	Sigma-Aldrich
Phosphate buffered saline (PBS)	Sigma-Aldrich
Polyacrylamide	Sigma-Aldrich
Serine	Sigma-Aldrich
Sodium chloride	Sigma-Aldrich
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Sodium phosphate monobasic	Sigma-Aldrich
Tetrahydrofuran	Sigma-Aldrich
Tetramethylethylendiamin (TEMED)	Sigma-Aldrich
Trifluoroacetic acid (TFA)	Sigma-Aldrich
Tris base	Sigma-Aldrich
Zinc chloride	Sigma-Aldrich

2.2 Main trial scheme

The exercise of this thesis was split into two main categories. On the one hand the production process of fish muscle peptides and on the other hand the quantitative and qualitative biochemical analyses of intermediates and final products.

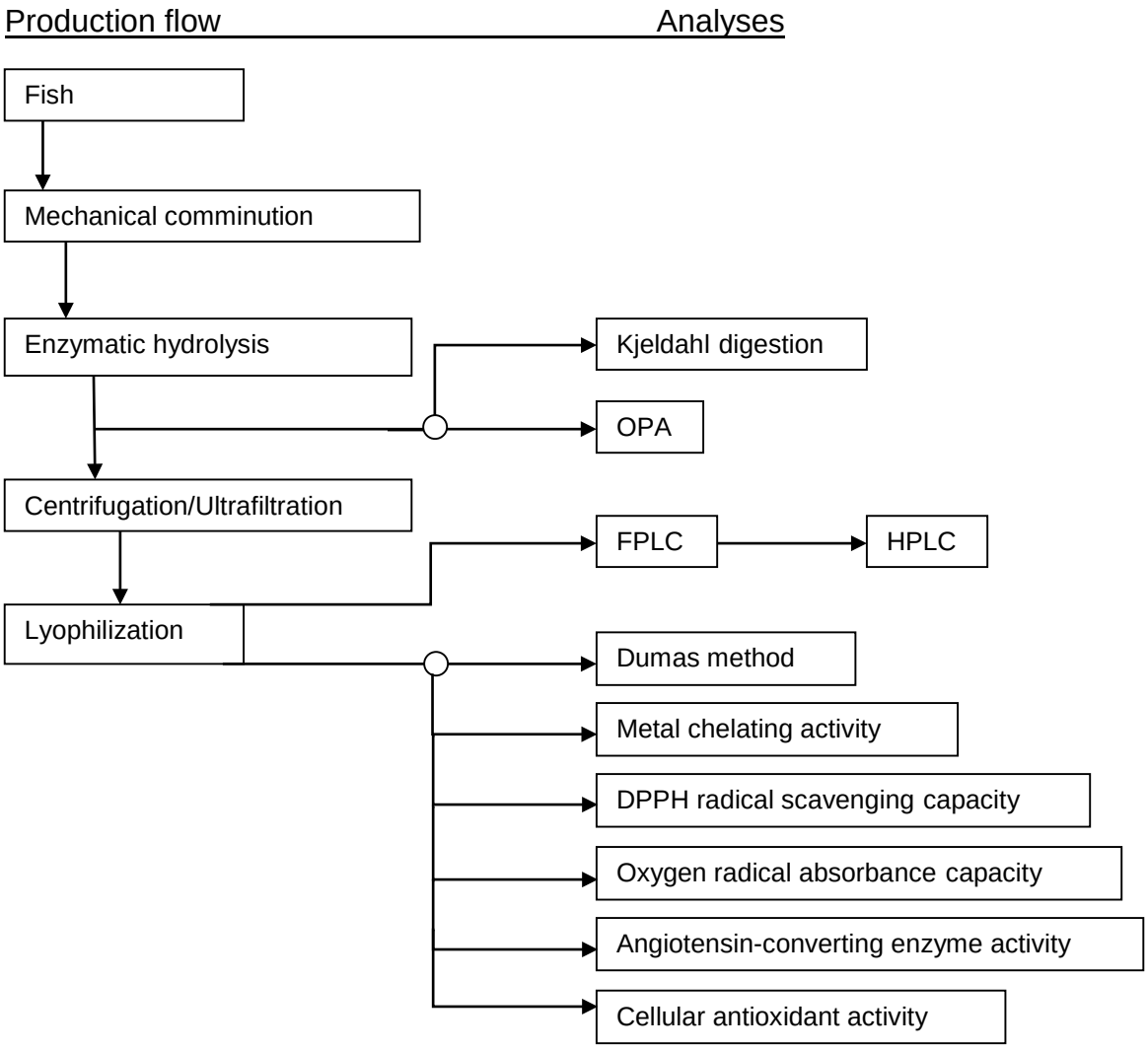

Fig.2.1: The main production steps and biochemical analyses used in this thesis.

2.3 Manufacturing process

2.3.1 Raw Material

As sample to pass through the production flow and examine quantitative, qualitative and bioactive properties from fish muscle peptides of a North Atlantic - marine organism, the fish species *Cyclopterus lumpus* (lumpfish) was used as raw material. The fish was cleaned and skinned delivered as fillets in a vacuum packed plastic bag and unfrozen at room temperature overnight.

2.3.2 Fish mince

The lumpfish filets were cut into slices and the cartilage and bones carefully removed. The fish pieces were put into a blender (Hallde VCB-62) and chopped for around 5 seconds to obtain well homogenized fish mince. The final mince was blended with preheated water and divided into equal amounts for each variant (100ml). Average protein content in lumpfish (8.5%) was used to dilute the mince with the required preheated water amount to 5.0% of protein in the solution. Depending on the enzyme stability and activity the pH of each variant subset was separately adjusted with 2M HCl to achieve 4.5 or 5.5. All net weights-, added volumes and the gross weight were documented and distinctive labelling done.

2.3.3 Enzymatic Hydrolysis

The samples were according to their test variant put into shakers (Innova 4400 incubator shaker) and heated up to the designed temperatures (35, 40, 45 and 50°C) while swirling at 130rpm. After approximately 1h, when the samples had reached their final temperature the enzymes could be added. The enzyme to substrate ratios (E/S) used were 1:50 and 1:100. For this trial 3 different proteases were tested (Tab.2.3). From now on the temperature and pH-value were checked and readjusted with either 2M NaOH or 2M HCl every hour during the hydrolysis process to keep the conditions as stable as possible. In order to achieve less and stronger hydrolysed products with the intension to find an optimal setting in regard to the bioactivity results of the fish hydrolysates, different hydrolysis times (1h, 2h, 3h and overnight) were applied. To guarantee an exact time of hydrolysis,

subsequently the solutions were filled in heat resistant plastic bags and heated up to 90°C in a water bath (Julabo 33) for 20min and thus enzymes completely inactivated. The heat treated samples were put on ice and cooled down for centrifugation.

Tab.2.3: Listed are the proteases used for the enzymatic hydrolysis of lumpfish.

Enzyme	Supplier
Protamex®	Novozymes
Protease M "Amano"	Amano Enzyme Inc.
Protease P "Amano" 6	Amano Enzyme Inc.

2.3.4 Centrifugation and Ultrafiltration

The first step of purification was to centrifuge the crude lumpfish hydrolysates with 10,000 x g for 20min at 4°C (Beckmann coulter Avanti J-20XPI) to get rid of the denatured enzymes and non-degraded bigger proteins. A floating layer of fat was removed by pouring the centrifuged solution over a fourfold pleated cheese cloth. Around 21g of each fish hydrolysate variant was separately collected for OPA and Kjeldahl measurement. The remaining volumes were cautionary stored at -24°C and ready for a more selective purification step.

The Ultrafiltration was carried out with centrifugation tubes with a 3kDa sized inner membrane (Amicon Ultra- 15 Centrifugal Filter Units). Therefore the frozen lumpfish hydrolysates were defrosted overnight in the fridge and spun with 4000 x g in a swinging bucket rotor centrifuge (Beckman TJ25) at 4°C until approximately 90% of the retentate had been recovered. The final permeate, consisting of molecules lower than 3kDa, were put back again into the freezer (-24°C) as pre-processing for freeze drying.

2.3.5 Lyophilisation

The frozen fish hydrolysate solutions were placed onto freeze dryer shells and steadily a vacuum of 0.001mbar generated. The treatment was running at -54°C for

1 week with the goal to achieve a virtually water-free peptide powder (1-4% final water content). Now the better and longer storable lumpfish hydrolysates were used to characterize their biochemical properties via chromatographic and bioactive measurements.

2.4 Biochemical Analyses

2.4.1 Determination of Degree of Hydrolysis (DH)

The DH is defined as the proportion of all hydrolysed peptide bonds (h) and the total number of peptide bonds (h_{tot}) per protein equivalent.

$$DH(\%) = (\frac{h}{h_{tot}}) \cdot 100$$

The degree of hydrolysis (DH%) was determined using the o-phthaldialdehyde OPA method described by Nielsen et al, 2001. Therefore 1ml of hydrolysed lumpfish muscle proteins was diluted with dH_2O into 100 or 250ml Erlenmeyer flasks (dependent on the intensity of the enzymatic hydrolysis). The reagent was prepared dissolving 7.620g Borax and 200mg SDS at around 45°C with 150ml dH_2O. OPA was separately dissolved in 4ml ethanol 94% and added with 176mg DTT by rinsing with dH_2O to the Borax-SDS solution. Finally filled up to 200ml and properly mixed with a magnetic stirrer (final OPA reagent). As standard 50mg serine were prepared in a 500ml flask (0.9516 meqv/L).

For the measurements, quadruplicates of OPA reagent for each sample, blank (dH_2O) and standard were filled into reaction tubes. 400µl of blank, then standard and finally sample were added to the 3ml OPA reagent, mixed for 6 seconds and left standing to react for 2min. Subsequently put into the spectrophotometer (Ultrospec 3000 pro) and read at 340nm. A typical value for the blank was 0.04 and for the standard 0.8. The OD values of the samples were minded to be less than 1. To determine the number of hydrolysed peptide bonds (h) the following calculation was done.

$$Serine\text{-}NH_2 = \frac{OD_{sample} - OD_{blank}}{OD_{standard} - OD_{blank}} \cdot 0.9516 \frac{meqv}{L} \cdot 0.1 \cdot \frac{100}{X} \cdot P$$

Serine-NH_2: meqv serine NH_2 / g protein

X: gram sample

P: protein % in sample

0.1: Sample volume in liter (L)

For the total number of peptide bonds (h_{tot}) the constant for the specific raw material "fish" was used to complete the %DH calculation.

2.4.2 Protein determination

The protein content in the hydrolysed fish samples after centrifugation was determined using the Kjeldahl method. The analysis was conducted from Matis employees in the department for analysis and consulting. The protein content in the hydrolysed fish samples after lyophilisation was determined using the method of Dumas. The measurements were implemented from Matis ohf. at Mýrargata 10, 740 Neskaupstaður. The Kjeldahl protein content was used to calculate the number of hydrolysed peptide bonds (h), whereas Dumas was done for bioactivity determinations.

2.4.3 Polyacrylamide gel electrophoresis

SDS-PAGE was used to identify the molecular weight of the purified fish peptide samples. Precast linear gradient (4-15%) polyacrylamide gels (Ready Gel Tris-HCl Gel #161-1104) from BioRad appeared to be partly suitable to detect the low molecular weight peptides, whereas self-made 16, 18, 20 and 25%resolving gels couldn't achieve enough resolution and band separation. 20µl sample solution (containing 50µg peptide) were mixed with 6µl of 5x sample buffer, boiled for 5min at 95°C and centrifuged prior loading it into the wells. The gels were run in a 1x running buffer with a constantly applied voltage of 200V (Amersham Pharmacia Biotech electrophoresis power supply). A peptide standard (polypeptide SDS-PAGE molecular weight standard #161-0326) functioned as molecular weight ladder for the samples. The gels were stained and destained according the instructions of the peptide standard protocol (Bio-Rad Laboratories, 2000).

2.4.4 Liquid Chromatography

2.4.4.1 Fast Protein Liquid Chromatography (FPLC)

For estimating the peptide size and composition in the sample a FPLC (ÄKTApurifierTM System) was conducted using a size exclusion column. The optimal fish hydrolysate amount of 2.5mg, to closely not maxing out the detector signal, was dissolved in phosphate buffer to a concentration of 2.5mg/0.9ml. Next the sample solution was before injection filtered through a 0.45µm syringe filter to remove potential interfering particles with bigger size. A phosphate buffer was made out of dipotassium phosphate and prepared in a blue cap bottle. The pH-value of the buffer was adjusted to pH 7.5 with 1M HCl to improve the solubility of the fish muscle peptides. To maintain a long column life, ddH2O was used as salt solvent and a filtration (0.22µm] was done prior chromatography start. The column (Superdex Peptide HR 10/30) and the whole system were washed with one column volume (24ml) of phosphate buffer. If the column was stored with ethanol, two column volumes were used for equilibration. Once stable curve shapes were observed with the equilibration process, the system is ready to start a chromatographic run by choosing a pre-programmed method on the UNICORN™ Software Version 3.2. The most run affective parameters were adjusted with the following conditions:

- flow rate: 0.5ml/min
- duration: 2 column volumes
- backpressure limit: 1.1 mPa

The prepared samples were injected with a syringe into the loading chamber directly connected to a 1ml injection loop. The running program was automatically loading the column to a given time with the injected sample by pressing the buffer through the injection loop. The UV-detector (Monitor UV 900) was scanning the flow through in the flow cell with multiple wavelengths and the signals were recorded as a graph and table form with the Unicorn program. The high variety of recorded parameters (e.g. 3 different wavelengths, conductivity, pressure, pH-value) enabled an indicatory first overview of the sample composition.

2.4.4.2 Reversed-phase high performance Liquid Chromatography (RP-HPLC)

The sample injection was ensued in liquid phase via a syringe pressing the sample into the injection loop standing on mode “load”. Therefore around 60mg of freeze dried fish hydrolysate samples were dissolved in 1ml of buffer A (0.1% (v/v) TFA) and filtered through a 0.45µm syringe filter prior injection. The buffer B (75:25/ACN:buffer A (v/v)) served for eluting the peptides off the stationary phase. All solvents without HPLC grade manufacturing were filtered through a 0.22µl filter prior use. The buffers were prepared in a sterile blue cap bottle in which the inlet pipe was sucking the liquids through an equipped filter into the HPLC. The security guard was connected to the mixer outlet and mounted onto the C18 column whose exit connected the UV-Vis detector. Subsequently the set HPLC (Kontron instruments) and especially the column (Kinetex™ C18, 250mm) was equilibrated with buffer A for 10min and checked for leakproofness. After the baseline was showing a stable and flat line at around zero absorbance the running conditions could be set as follows:

- flow rate: 1ml/min
- duration: 120min
- detection wavelength: 220nm
- ambient temperature
- gradient:

Tab.2.4: A linear gradient from 5-45%B was used to elute the peptides from their interaction to C18 wall.

Duration time [min]	Buffer B [%]
0	5
120	45

Once the HPLC settings are done it is time for the manual sample injection. The already prepared sample is put into the 20µl injection loop and with the lever turn to “inject” the program starts to run and record. Figure 1 illustrates an example of an elution profile of diverse natural fish peptides obtained from an RP-HPLC run under specific conditions. The data points are exported as a text file and peak calculations can be done in excel or directly in the chromatographic program.

2.4.5 Antioxidant assays

2.4.5.1 Metal chelating activity

One antioxidant assay was carried out using the property of peptides forming soluble complexes with iron. Fe_2^+ chelating activity was detected by analysing the formation of the Fe_2^+ ferrozine complex. Beforehand 0,2 mM ferrous chloride and 0,5 mM ferrozine were freshly prepared and kept in the dark. The reagents were 10 x diluted and the lumpfish samples adjusted to a concentration of 1mg/ml. It was then pipetted according the table 2.4.5 with water as control.

Tab.2.4.5: Listed is the pipetting order with the corresponding volumes.

Blank	Control	Sample
100 µL water	100 µL sample	100 µL sample
50 µL $FeCl_2$	50 µL $FeCl_2$	50 µL $FeCl_2$
100 µL ferrozine	100 µL water	100 µL ferrozine

Subsequently the plate was stood into the shaker and left at low rotation for 30min. After the complex forming process between Fe(II) and ferrozine, which generated depending on the degree of oxidation a violet to greenish colour, the samples were spectrophotometric analysed at 560nm using a microplate reader (POLARstar OPTIMA, BMG Labtech).

$$\text{Chelating activity (\%)} = \frac{A_{blank} - (A_{sample} - A_{control})}{A_{blank}} \cdot 100$$

Where *Ablank* is the absorbance of the blank, *Asample* is the absorbance of the sample and *Acontrol* is the absorbance of the control samples at 560nm.

2.4.5.2 DPPH radical scavenging capacity

In this assay, the purple DPPH was reduced by antioxidant (peptides) to the corresponding yellow hydrazine (Karadag, Ozcelik, & Saner, 2009). 100µl (1mg/ml) of fish hydrolysate solution was added to 900µl methanol and centrifuged for 10min with 2000rpm at 4°C. 150µl of the supernatant were then pipetted with 60µl of DPPH solution into an aluminium covered microplate. This was incubated at room temperature for 30min and read at 520nm (POLARstar OPTIMA, BMG Labtech).

$$\text{inhibition (\%)} = \frac{A_{blank} - (A_{sample} - A_{control})}{A_{blank}} \cdot 100$$

Where *Ablank* is the absorbance of the blank (ddH_2O), *Asample* is the absorbance of the sample and *Acontrol* is the absorbance of the control samples at 520nm.

2.4.5.3 Oxygen radical absorbance capacity (ORAC)

The ORAC assay determines the antioxidant inhibition of peroxyl-radical induced oxidation via H-atom transfer (Karadag et al., 2009). To test this reaction, 60µl of fluorescein (10nM) were pipetted into empty 96 well plates. Some of the wells were combined with 40µl phosphate buffer (10mM, pH 7.4) for a gain adjustment. The samples, blank (ddH_2O) and a standard (Trolox) were added to the fluorescein wells (10µl) and incubated for 15min at 37°C without shaking. The oxidation reaction was initiated mixing 30µl of AAPH (120mM) manually with a multi-channel pipette into all of the wells. Immediately the microplate was put into the POLARstar (BMG Labtech) and measurement started by exciting the solution with 484nm and recording the emission light at 520nm every minute for a time span of 100min. The area under the fluorescence decaycurve (AUC) was calculated by normalized curves using the following formula.

$$AUC = \left(\frac{f0}{f0} + \frac{f99}{f0}\right) \cdot 0.5 + \left(\frac{f1}{f0} + \ \ldots + \frac{f98}{f0}\right)$$

Where f0 was the fluorescence reading at the initiation of the reaction and f99 was the last measurement at minute 100.

2.4.5.4 Cellular antioxidant activity (CAA)

With this method the ability of the fish hydrolysates to prevent the formation of DCF by 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP)-generated peroxyl radicals in human hepatocarcinoma HepG2 cells was tested (Wolfe & Liu, 2007).
An intracellular antioxidant assay was performed on lumpfish hydrolysates using HepG2 cells maintained in Minimum Essential Medium α (MEMα), supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (50 units/mL), and streptomycin (50 µg/mL). Cells were incubated at 37 °C in a fully humidified

environment under 5% CO2, and HepG2 cells at passage 80-100 were used for the experiments. Cells were subcultured at 3-5 days intervals before reaching 90% confluence. The assay was carried out after HepG2 cells reached a cell density of 6×10^4/well using black 96-well plates (BD Falcon™) in 100 µL growth medium/well. Twenty four hours after seeding, 100 µL of DCFH-DA probe (1 µM in HBSS) was added to the cells and incubated at 37 °C in the dark for 30 min. Cells were then treated with different concentrations of lumpfish hydrolysates and incubated for 1 h at 37 °C. This was followed by the addition of 100 µl of AAPH (500 µM in HBSS) to the cultured cells after removal of the test compounds. Fluorescence readings (λexcitation = 493 nm, λemission = 527 nm) were recorded using the POLARstar OPTIMA (BMG Labtech) every 10 min for 2 h after addition of AAPH. Each plate included four replicates of control and blank wells: Blank wells contained cells exposed to only the DCFH-DA probe. The control consisted of cells with DCFH-DA probe and the AAPH peroxyl radical initiator added, but in the absence of test compounds.

2.4.6 Antihypertensive assay: ACE inhibitory activity

This assay was done to determine a potential antihypertensive activity of fish hydrolysates, where the conversion of the internally quenched fluorescent substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline via ACE to the fluorescent product o-aminobenzoylglycine could be used to monitor the mode of action with the POLARstar (nature protocol). For quantifying the ACE activity in fish hydrolysates, 20µl of sample and blank (50mM Tris-base buffer, pH 8.3 were pipetted in a 96-well microplate and subsequently mixed with 10µl ACE solution (1 unit ACE vial mixed with 2ml 100mM Tris-base + 2µM $ZnCl_2$, pH 8.3 and 2g glycerol give 12.5mU/ml ACE). 170µl of pre-heated (37°C, 15min) 0.88mM substrate working solution (N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly = FAPGG prepared in 50mM Tris-base + 1.125M NaCl, pH 8.3) was added and thoroughly mixed with the sample/ACE solution. The microplate was read with the POLARstar for 60min at 37°C with records every minute at 340nm.

$$\text{ACE inhibition } (\%) = (1 - \frac{\text{A}_{\text{sample}}}{\text{A}_{\text{blank}}}) \cdot 100$$

Where *Ablank* is the absorbance of the blank (50mM Tris-base buffer, pH 8.3) and *Asample* is the absorbance of the sample 340nm.

3 Results

3.1 Setting of enzymatic hydrolysis parameters

The parameters with a main influence on enzymatic hydrolysis were theoretically analysed and a plan constructed with many variants. The enzymes used were all granulated proteases from 2 different suppliers. Protamex, an endoprotease complex from *Bacillus* type, was purchased from Novozymes (Protamex - Product Sheet, 2001). Proteases M, a fungal acid protease from Aspergillus oryzae, and Protease P, derived from Aspergillus melleus, were manufactured from Amano Enzyme Inc. (Protease M "Amano", 2003); (Protease P "Amano"6, 2003).

Tab.3.1: Listed are the parameters for enzymatic hydrolysis including enzyme type, time, pH-value, enzyme substrate ratio [E/S] and temperature.

Enzyme	Time [h]	pH-value	E/S	Temperature [°C]
Protease M	1	5.5	1:100	40
Protease M	1	5.5	1:100	40
Protease M	3	5.5	1:50	50
Protease M	3	5.5	1:50	50
Protease M	12	4.5	1:50	35
Protease P	1	5.5	1:100	40
Protease P	3	5.5	1:50	50
Protease P	12	4.5	1:50	35
Protamex	1	5.5	1:100	40
Protamex	3	5.5	1:50	50
Protamex	12	4.5	1:50	35
Protease M + Protease P	2	5	1:50	45
Protease M + Protamex	2	5	1:50	45
Protease P + Protamex	12	4.5	1:50	35

The environmental conditions (pH-value and temperature) were chosen in regard to the working range of the proteases and different enzyme substrate ratios (E/S) and hydrolysis times were tested to determine the efficacy of the enzymes (Tab.3.1).

3.2 Protein analyses and determination of degree of hydrolysis

The biochemical analyses of lumpfish hydrolysate started after the enzymatic hydrolysis and the first purification step via centrifugation with the measurement of the protein content in solution. The sum of all organic nitrogen in the solution was determined using the Kjeldahl method, where the protein content can be calculated with a fish protein specific conversation factor. The results of the protein content in the fish hydrolysate solution were necessary to be able to calculate the DH after previous taken OPA measurements. Independently to the protein content in liquid form of the sample, it was also taken from the lyophilized fish hydrolysates to be able to calculate some bioactivity analysis results.

The longer hydrolyzed lumpfish samples (12h) with Protease M, Protease P and a combination of Protease P and Protamex revealed a relatively high DH of nearly 50%. Protease M and Protease P digestions over 3h and enzyme combinations for 2h showed a degree of hydrolysis of around 30-40%. The single Protamex hydrolysed samples, except Protamex 12h, and all other variants with 1h hydrolysis appeared to have a low hydrolysing potential with a DH of approximately 20% and below.

The protein analyses resulted in contents from 2.09 – 5.14% in liquid samples and 77.5 – 90.3% protein in the freeze dried lumpfish hydrolysates. The protein contents *wet* and *dry* did not relate to any enzyme type or hydrolysis time, respectively. There was also no obvious relation between protein content and degree of hydrolysis (Tab.3.2).

Tab.3.2: Shown are the corresponding protein contents and the degree of hydrolysis of the enzyme variant as a function of time, whereby protein content *wet* is referring to Kjeldahl digestion and protein content *dry* was observed with Dumas method. The table is ordered beginning with the sample with the highest DH.

Enzyme/time variant	Protein content *wet* [%]	Protein content *dry* [%]	DH [%]
Protease M 12h	3.98	88.3	48.4
Protease P 12h	3.96	90	46.4
Protease P + Protamex 12h	5.14	90.3	44.7
Protease P 3h	5.11	88	39.7
Protease M + Protease P 2h	3.98	87	36.9
Protease M 3h	3.16	84.9	34
Protease M + Protamex 2h	3.52	85.2	32.7
Protamex 12h	4.39	89.5	31.9
Protease P 1h	3.47	85	23
Protamex 3h	3.69	87.2	20.7
Protease M 1h	2.09	77.5	16.8
Protamex 1h	2.69	84.8	14.7

3.3 Chromatographic profiling of lumpfish hydrolysates

3.3.1 Peptide size estimation via FPLC

The hydrolysed and ultrafiltered (<3kDa) lumpfish samples were examined after lyophilisation for their yielding peptide size fractions. Size exclusion chromatography with a column suitable for peptides between 100 – 7000Da was applied on the ÄKTA*purifier*™ System. The hydrolysate powders were again liquidized, filtered and manually injected into a 1ml loop. The FPLC runs were performed with 0.5ml/min flow rate and peptide bond response recorded at 220 and 280nm.

As a representative extract of all 3 used enzymes, depicts Protease P the differences in received peptide sizes after 1h, 3h and overnight (12h) hydrolysis. The UV detector signals at 280nm generated distinctive curves within each sample run. Peptides produced after 1h hydrolysis were the first coming off the size exclusion column indicated in an rising peak after 10ml of flow through.

Subsequently the 3h Protease P variant appeared on the screen with a much higher later peak after 28ml passed mobile phase than the 1h variant. Similar shapes to the 3h sample, but with almost no absorbance value at 28ml, were observed with 12h hydrolysis (Fig.3.1).

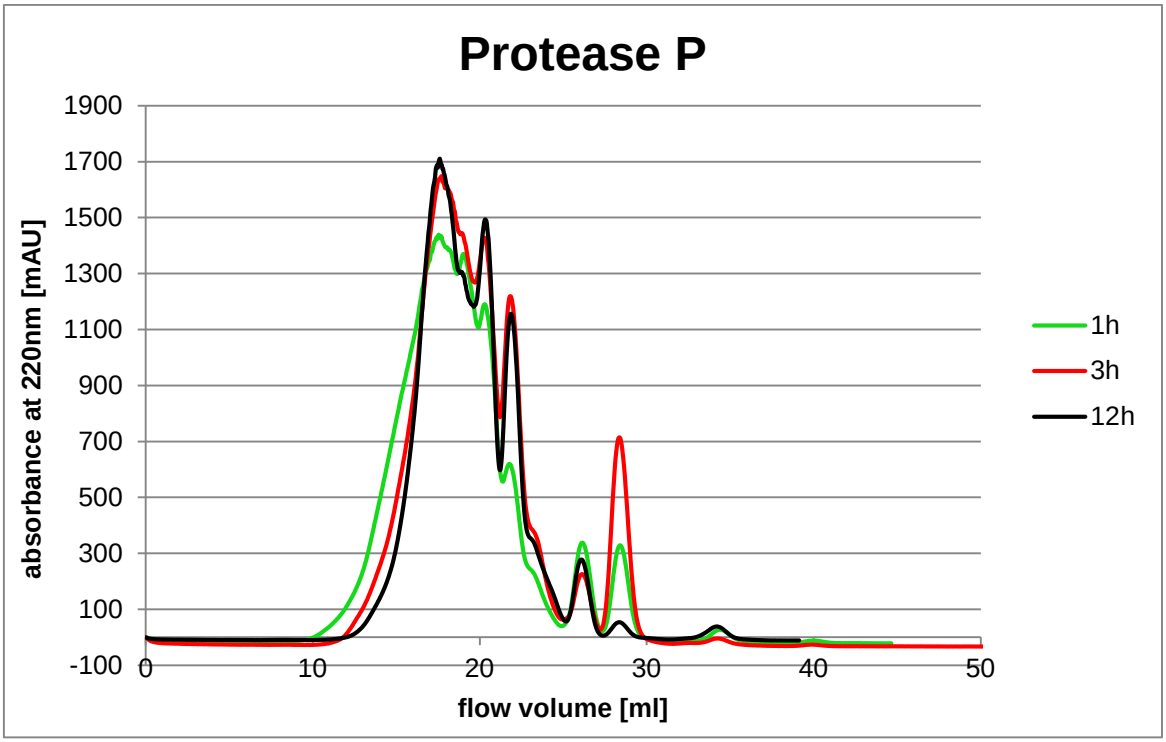

Fig.3.1: The graph illustrates the distinctive curves observed with Protease P hydrolysis after 1h, 3h and 12h. In green the curve with the 1h variant showing a comparative early peptide elution with a weaker absorbance when coming to the end of the run. The red line presents the absorbance values at 220nm of the hydrolyzed lumpfish after 3h with a standing out peak after around 28ml flow volume. A hydrolysis with Protease P for 12h (black) behaved on the ÄKTA system similar to the 3h variant.

The differences in peptide generation on lumpfish muscle proteins within the used proteases have been made visible with an overlay of the 3h enzyme variants. Protamex revealed the earliest (~10ml) and strongest (~15ml) peptide signals at 220nm with smaller peaks in the end of the chromatographic run. Protease P and Protease M shared nearly the same curve behaviour indicating a peptide composition with similar sized amino acids chains. Referring to the general size exclusion principle (Chapter 1.6.3), which states that the bigger particles will not enter the beads and therefore coming off the column earlier, it can be assumed that Protamex produced bigger peptides due to the comparative high absorbance values (2100mAU) in the end of the first third of the run (13 – 17ml) (Fig.3.2).

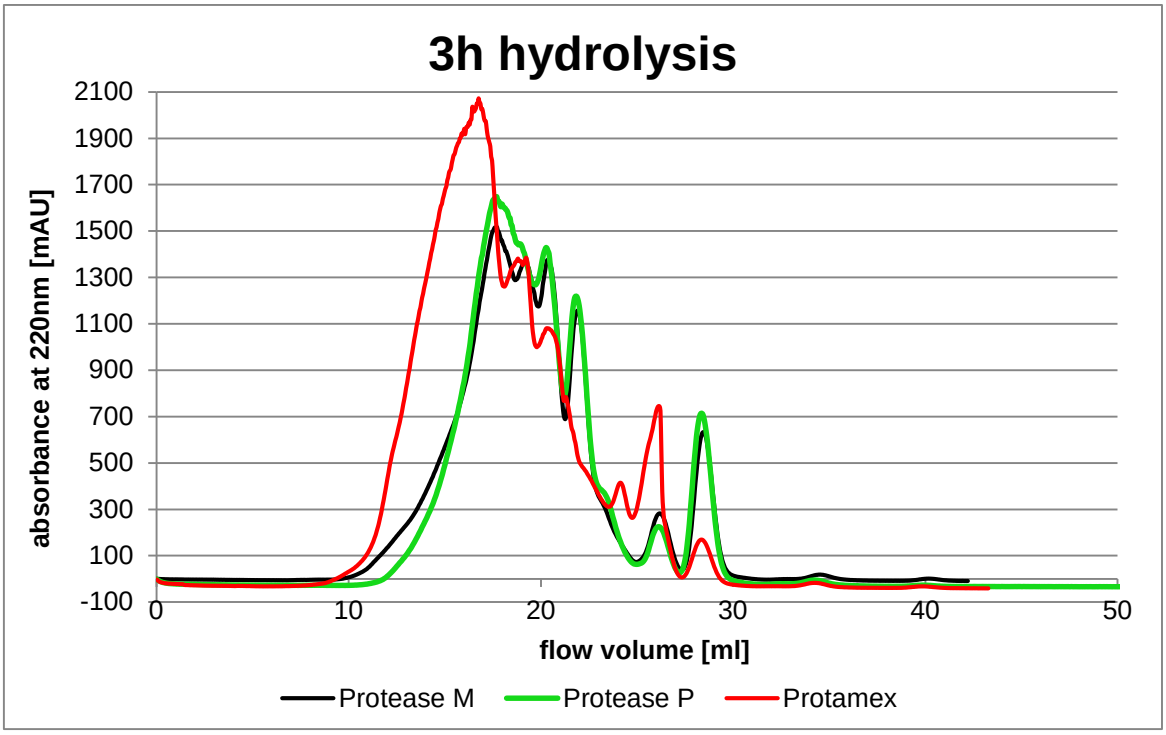

Fig.3.2: Fig.3.2: The graphical overlay gives a first impression of the cutting pattern of Protamex, Protease P and Protease M after 3h hydrolysis on lumpfish muscle proteins. Protease M and Protease P presented with a black and a green curve showed similarity in their chromatographic profile with a relatively late peptide elution. To the contrary is the in red indicated Protamex curve characterized by an early and high absorbance.

3.3.2 Hydrophobic interaction profile of peptides based on RP - HPLC

For figuring out the hydrophobicity of the peptides from the various lumpfish hydrolysates a reversed – phase HPLC was performed. Similar to FPLC was the hydrolysate powder dissolved in buffer, injected into a 20µl loop and loaded onto a C_{18} column. The hydrophobic interacting peptides were eluted with an acetonitrile – TFA water mixture (75:25) using a linear gradient from 5-50%. In conjunction with the elution gradient contributed a constant flow rate of 1ml/min and 120min retention time to an adequate peak distribution with a high resolution. The UV measurements were taken at 220nm and known peptide standards served as a chromatographic ruler for the samples (Fig.3.3).

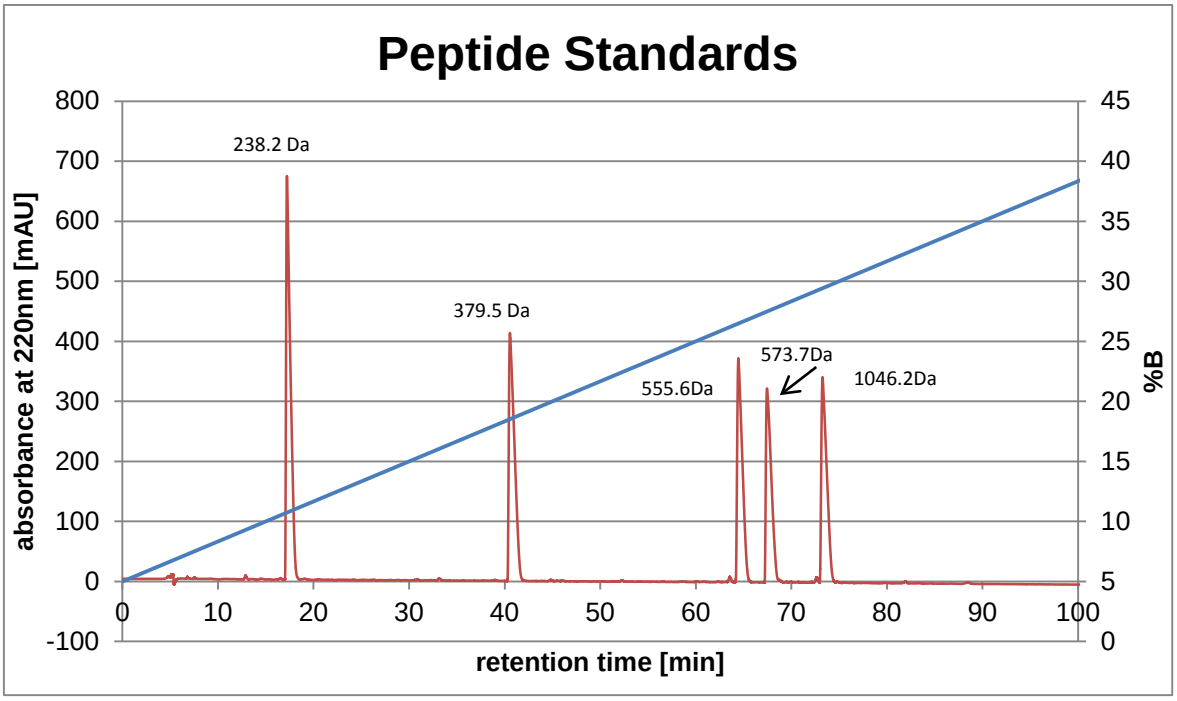

Fig.3.3: The red line of the chromatogram depicts the occurred peaks absorbed from the UV-VIS detector at 220nm from known peptides. The various peptides range from dipeptides to peptides with 8 amino acids. The blue line indicates the set gradient of the organic phase (solvent B in %) GLY-TYR = 238.2Da, VAL-TYR-VAL = 379.5Da, Leucine Enkephalin (TYR-GLY-GLY-PHE-LEU) = 555.6Da, Methionine Enkephalin Acetate (TYR-GLY-GLY-PHE-MET) = 573.7Da, Angiotensin II Acetate (ASP-ARG-VAL-TYR-ILE-HIS-PRO-PHE) = 1046.2Da.

To see the influence of hydrolysis time on the peptide generation, Protease M was used as a representative example for all enzymes. It was generally observed that the 1h variants didn't show much absorbance at all. One higher peak (350mAU) after around 6min and 3 smaller deflections at 12, 15 and 37min (~100mAU) were specifically obtained with Protease M 1h. The variant with 3h hydrolysis revealed a rather high absorbance at 220nm on 2 distinctive peaks after 14min (450mAU) and 33min (250mAU). Two equally high peaks (450mAU) were recorded with Protease M hydrolysed for 12h after 15 and 38min retention time indicating a comparatively huge amount of small hydrophobic peptides (Fig.3.4).

The different cutting pattern of Protease M, Protease P and Protamex on lumpfish muscle proteins was also detected with reversed-phase liquid chromatography. A graph comparing the chromatographic profiles of 3h hydrolysed samples is illustrating the significant difference, especially of Protamex versus the others (Fig.3.5).

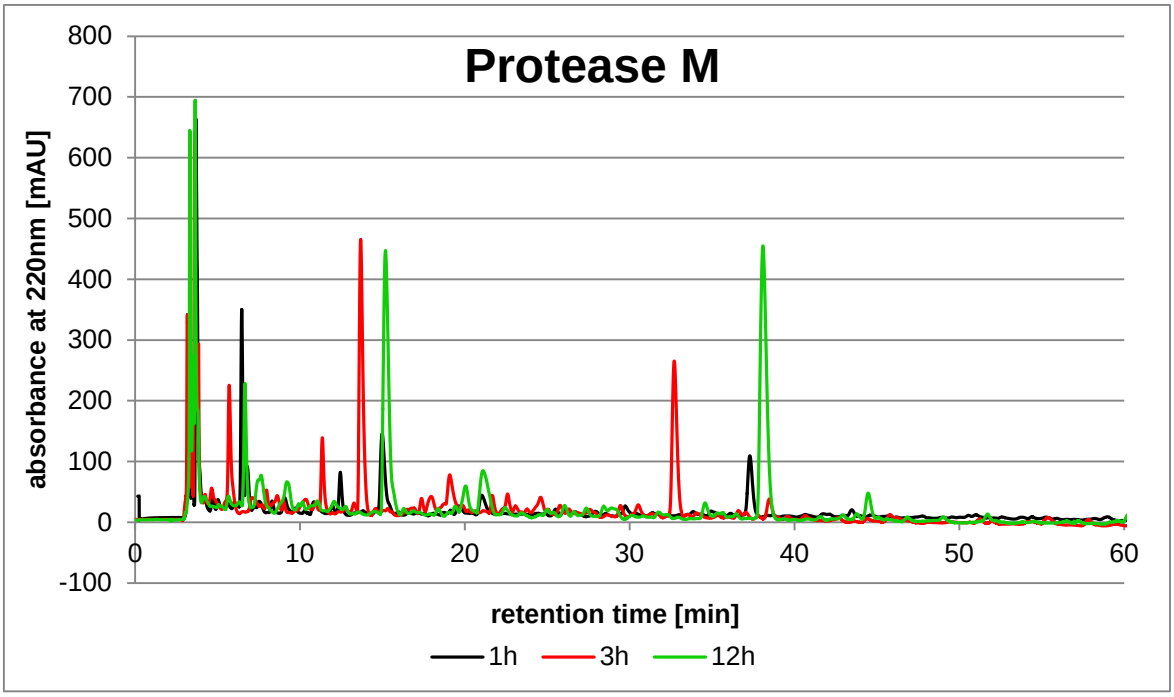

Fig.3.4: The curves gained with RP-HPLC are representing peptide fractions mainly according to their hydrophobic potential and partly to the size. The chromatographic profile obtained with Protease M is reflecting a general trend among other enzymes of few hydrophobic peptides after 1h hydrolysis (black line) and significant more peptide signals after 3h and 12h hydrolysis (red and green line).

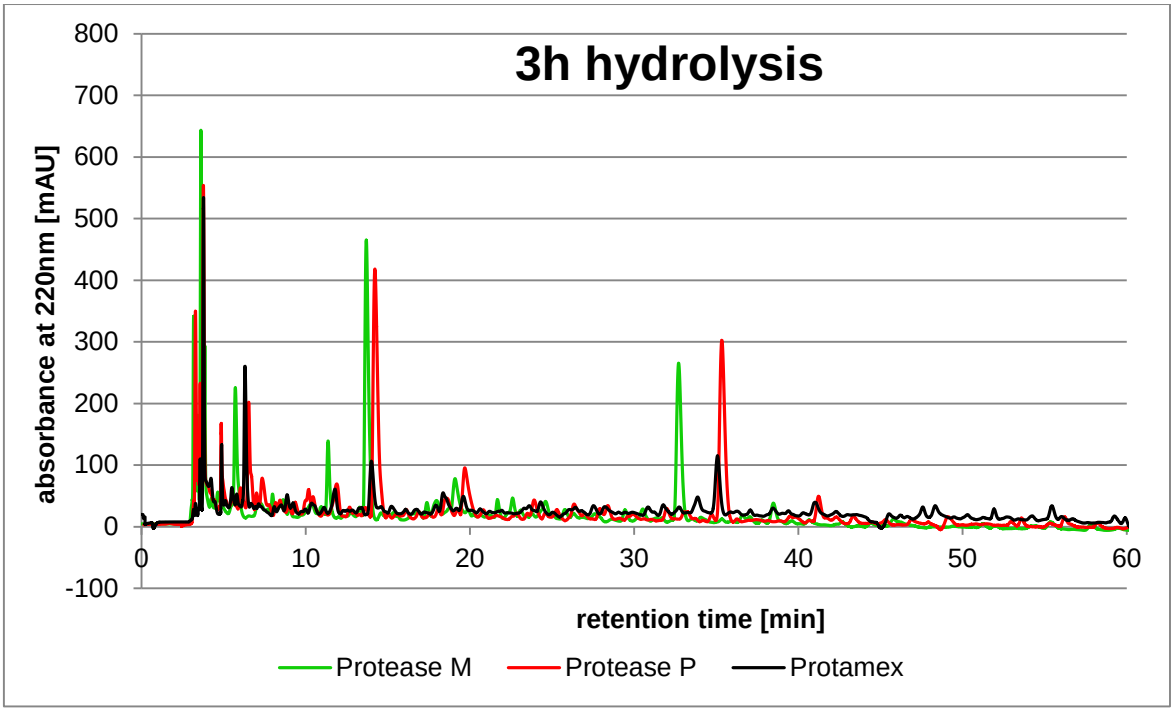

Fig.3.5: The different cutting pattern of the proteases after 3h hydrolysis is shown in this illustration. Protamex recordings (black) disclose with weak signals along the run a minor amount of hydrophobic peptides. Higher UV-Vis deflections at Protease P and Protease M may portend to more and stronger hydrophobic amino acids in the peptide chain.

Lumpfish powder hydrolysed with Protamex signalled only low absorbance values (~50-100mAU) on the hydrophobic HPLC column. By contrast Protease M and Protease P achieved similar high and well distributed peaks at around 15 and 35min (250-450mAU). The unsteady UV deflections and barely distributed peaks between 3 – 5min were an indication for hydrophilic or neutral peptides and other compounds with an absorbance spectrum at 220nm, which were not interacting with the hydrophobic stationary phase and thus washed out at first.

3.4 Antioxidant potential of lumpfish hydrolysates

3.4.1 Affinity of hydrolysates to bind iron

In order to determine secondary antioxidant ability of lumpfish hydrolysates ferrous was used as chelating metal. Unlike to direct antioxidants is the mechanism of secondary antioxidants not determined by forming more stable products with free radicals, but rather slowing down the oxidation rate of metal prooxidants by building complexes (Končić, Barbarić, Perković, & Zorc, 2011).

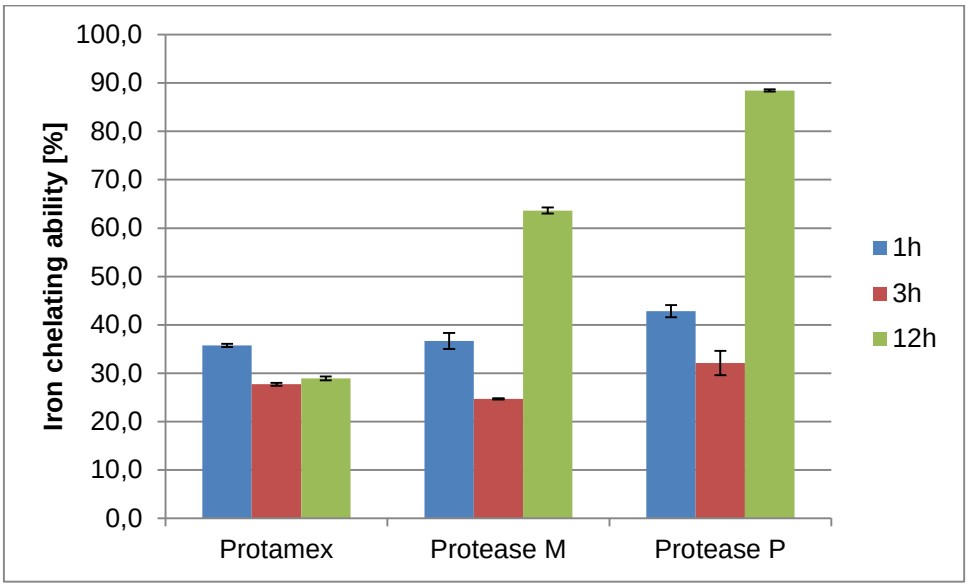

Fig.3.6: This figure illustrates the ability of lumpfish hydrolysates to form complexes with iron and thus acting as a secondary antioxidant. The samples with the highest metal chelating were Protease M and Protease P 12h with neglectable standard deviations.

All lumpfish samples were able to chelate iron to a certain extend. The highest metal chelating ability was observed after 12h hydrolysis with Protease P (~90%) and

Protease M (~65%). The affinity of samples hydrolysed with Protamex 12h for ferrous ions was relatively low in comparison to the other proteases. Interestingly all 3h variants showed the lowest and nearly the same iron chelating ability in the analysis. A little bit better chelators than 3h samples seem to be the fish powders hydrolysed for 1h displaying a metal chelating ability of around 40% (Fig.3.6).

3.4.2 Ability of hydrolysates to reduce DPPH

DPPH is a representative free radical to those in the human body and due to its high molar extinction coefficient at 520nm a useable tool to determine free radical scavenging ability of hydrolysates. After reaction between DPPH and a potential antioxidant the colour of the solution diminishes and the difference is recordable with a spectrophotometer.

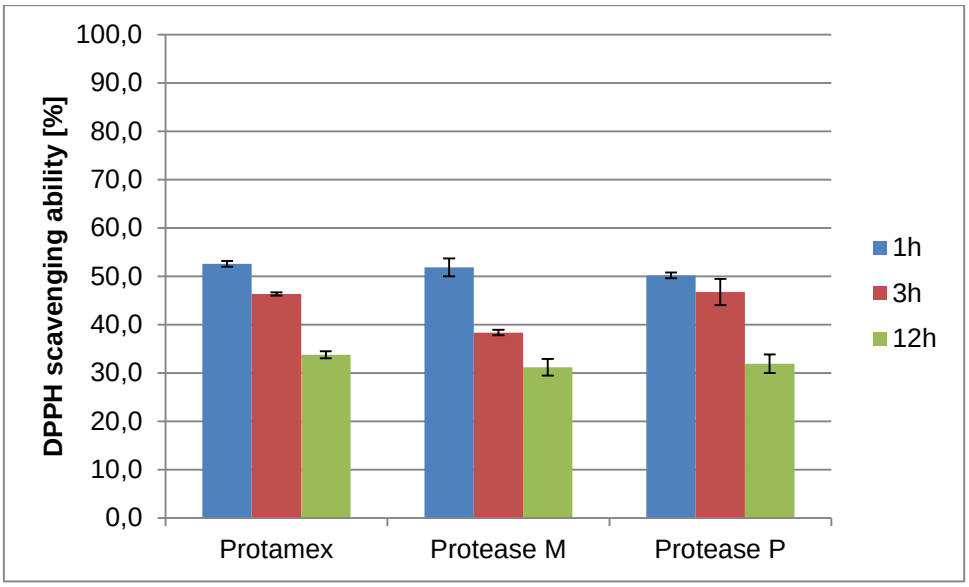

Fig.3.7: The ability to scavenge DPPH free radicals is presented in this bar chart. It shows an overall trend that shorter hydrolysed lumpfish muscle proteins, indifferent to the protease, review a stronger radical scavenging ability to DPPH than extended hydrolysis.

Investigated lumpfish samples with a hydrolysis time for 1h demonstrated remarkable DPPH radical scavenging ability higher than 50%. The antioxidant potential for DPPH went gradually downhill with expanded hydrolysis time. The reducing ability of hydrolysates towards the free radical was equally strong within the used enzymes (Fig.3.7).

3.4.3 Capacity of hydrolysates to absorb free radicals

The oxygen radical absorbance capacity of hydrolysates was determined with artificially generated peroxyl radicals from AAPH. These radicals react with a fluorescent probe over a specific time manner thereby loosing intensity of fluorescence, which indicates the damage on the fluorescent substance triggered by the peroxyl radical. Trolox, water soluble derivate of vitamin E, functions in that assay as standard and relational magnitude as a very strong antioxidant.

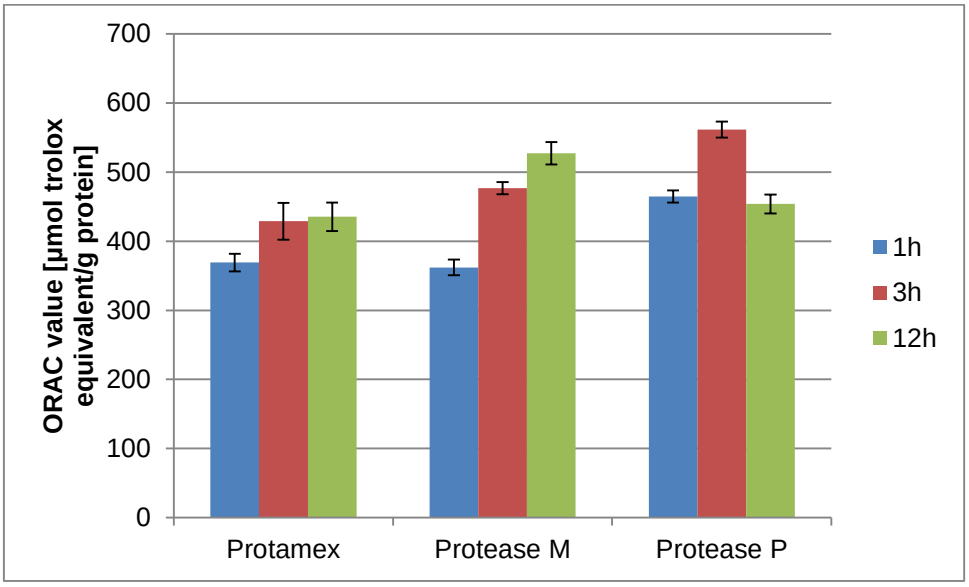

Fig.3.8: The capacity of lumpfish peptides to absorb oxygen radicals is illustrated in this graph with implicated standard deviation. In green the overnight variant (12h), red symbolizes 3h hydrolysis and blue 1h. The ORAC value is defined as µmol trolox equivalent per gram protein.

Conversely to the DPPH scavenging ability revealed the 1h variants the lowest antioxidant effect in the ORAC assay. The highest values were obtained from Protease P 3h and Protease M 12h, where approximately 550µmol trolox is needed to achieve the same radical absorbance capacity as 1g of protein. The samples hydrolysed with Protamex could not reach the same high antioxidant effect as the Amano proteases and thus characterized as relatively weak oxygen radical absorbing peptide powders (Fig.3.8).

3.4.4 Antioxidant activity of hydrolysates in human liver carcinoma cells

This assay measures the ability of hydrolysates to prevent the oxidation of DCFH-DA to highly fluorescent DCF induced by free radicals in human hepatocarcinoma HepG2 cells. The antioxidant effect of the hydrolysates is proportional to the generated fluorescent intensity. The higher the fluorescent signal the lower the antioxidant potential of the sample (Cell Biolabs, Inc., 2013).

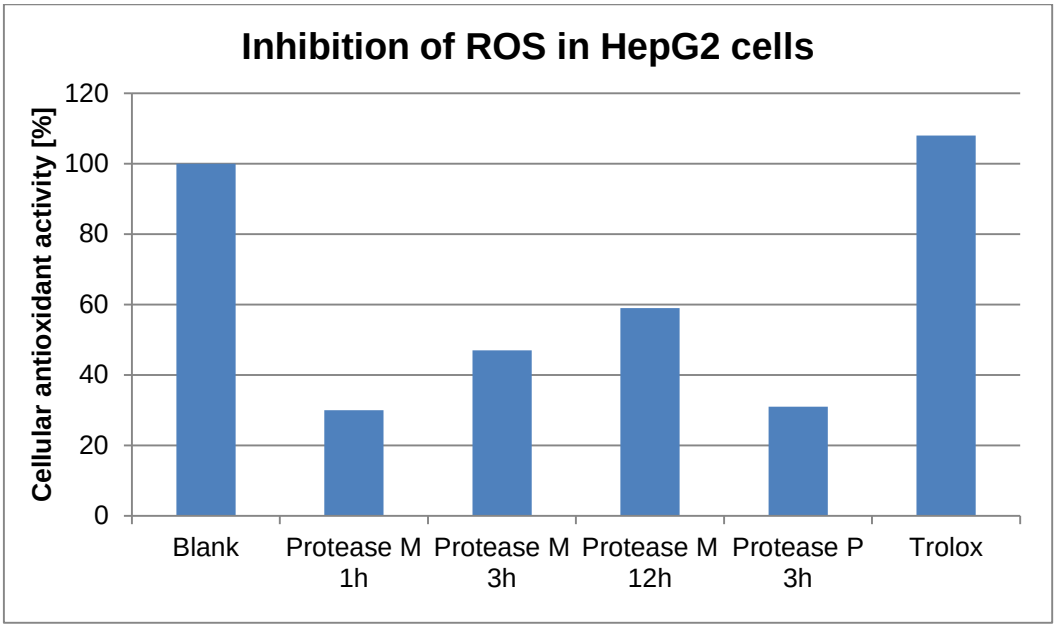

Fig.3.9: Effect on intracellular ROS level induced by AAPH in HepG2 cells by samples PM 3h, PM o/n, PP 3h and PM 1h. Blank consisted of cells exposed to only the DCFH-DA probe. The control consisted of cells with the DCFH-DA probe and the AAPH peroxyl radical initiator but in the absence of lumpfish hydrolysate (CAA = 0%, not shown). Trolox was used as positive reference substance with an excellent antioxidant activity (108% if assuming that blank is 100%).

Four of the lumpfish hydrolysate samples were tested for cellular antioxidant activity. Thereby showed Protease M 12h the highest antioxidant activity with 41% inhibited ROS radicals. In comparison to Protease P generated a 3h hydrolysis with Protease M more cellular antioxidant peptides. The effect of the strong antioxidant Trolox was equitable to the blank.

3.5 Antihypertensive potential of hydrolysates via ACE inhibition

The principle of this method lies in the hydrolysis of the intramolecular quenched tripeptide o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline via the action of ACE to the fluorescent product o-aminobenzoylglycine.

Tab.3.3: The effectiveness of lumpfish hydrolysates to inhibit ACE to 50%.

Enzyme variant	IC_{50} [mg/ml]
Protease P 3h	2.5 ± 0.0
Protease M 3h	4.0 ± 0.1
Protease M 12h	4.2 ± 0.0
Protease M 1h	7.6 ± 0.4

The reaction can be kinetically recorded at 340nm. For ACE inhibitory activity the same 4 sample as for CAA were tested. A enzymatic hydrolysis with Protease P for 3h may produced peptides with a comparative high ACE inhibitory potential (IC_{50} = 2.5), whereas Protease M hydrolysates couldn't reach such a low half maximal inhibitory concentration.

4 Discussion

Lumpfish protein hydrolysate shows antioxidant and antihypertensive properties. The bioactivity of the hydrolysate was primarily triggered by small peptides produced via enzymatic hydrolysis (Najafian & Babji, 2011). The type of enzyme, the degree of hydrolysis and finally the resulting peptide size and composition were the observed and documented influencing variables on the bioactive effects of the lumpfish hydrolysate.

An extended hydrolysis time led to a higher degree of hydrolysis, which was noticeable for all applied proteases. In spite of the fact, that the enzymes were all mainly developed for hydrolysis of food proteins, clearly visible differences in DH and bioactivity could be found. Protamex generated the lowest DH in all time frames (1h, 3h, and 12h) compared to the others. It turned out that Protease P had the highest reaction rate in catalysing lumpfish muscle proteins into smaller peptide units. With regard of the enzyme activity range it was surprising that the slightly alkaline Protease P showed better hydrolysing properties than the more acidic Protease M, since, except for the overnight (12h) variant, the pH during hydrolysis was adjusted to 5.5. An attempt to accelerate the time of hydrolysis by using 2 enzymes in combination was achieved with Protease M + Protease P 2h comparing with Protease M 3h. Taking the enzyme reaction curve into consideration it can be concluded that the enzyme efficacy is declining with time, which may reason from a lower substrate concentration in the end of the hydrolysis.

Assays performed to check potential antioxidant activity in lumpfish hydrolysate showed throughout positive results. By reason of plenty established antioxidant assays with different measuring principles, a broad range of indicatory antioxidant effects on lumpfish hydrolysate could be generated. Hydrolysates produced with Protease M and Protease P overnight (12h) were the most suitable to form complexes with ferrous and thus temporary inactivated reactive iron. Unlike the remarkable metal chelating ability of longer hydrolysed samples, the 1h variants demonstrated the comparative best DPPH radical scavenging properties. ORAC,

another in vitro assay, confirmed differences in antioxidant activity within enzymes and disproved a linear correlation between DH and antioxidant intensity. A few samples were tested for in vivo (HepG2 cells) antioxidant effects and revealed up to 59% ROS inhibition (Protease M 12h), which is encouraging for preclinical studies.

Tab.4.1: All tested variants with their corresponding degree of hydrolysis and bioactivity. DH (degree of hydrolysis), MC (metal chelating activity), DPPH (DPPH radical scavenging capacity), ORAC value (oxygen radical absorbance capacity), CAA (cellular antioxidant activity), ACE (angiotensin-converting enzyme inhibitory activity)

Enzyme variant	DH [%]	MC [%]	DPPH [%]	ORAC value	CAA [%]	**ACE** [IC_{50}]
Protease M 1h	16.8	36.7	51.8	362	30	7.6
Protease P 1h	23	42.8	50.2	464.7		
Protamex 1h	14.7	35.7	52.6	369.1		
Protease M 3h	34	24.7	38.4	476.7	47	4.0
Protease P 3h	39.7	32.1	46.7	561.7	31	2.5
Protamex 3h	20.7	27.7	46.3	429		
Protease M 12h	48.4	63.6	31.2	527..3	59	4.2
Protease P 12h	46.4	88.4	31.9	453.9		
Protamex 12h	31.9	28.9	33.8	435.4		
Protease M + Protease P 2h	36.9	33.8	37.2	474.6		
Protease M + Protamex 2h	32.7	31.1	45.1	448.9		
Protease P + Protamex 12h	44.7	58.1	32.1	494.2		

Peptides derived from lumpfish showed not only antioxidant activity, but also blood pressure lowering potential by inhibiting angiotensin-converting enzyme (ACE). An application rate of 2.5mg/ml of Protease P 3h could inhibit ACE to 50%, which was alike to the ORAC results the most effective sample (Tab.4.1).

For having more insights into the hydrolysate composition liquid chromatography was used to detect possible reasons for the measured bioactivity results. The size exclusion results (Fig.3.1, 3.2) performed with FPLC uncovered differences in enzymes used and hydrolysis time, whereas 1h variants and Protamex samples started earlier with the peak formation indicating larger peptides. Especially Protease M and Protease P created more and higher peaks in the end of the run symbolizing a higher concentration of smaller peptides in the hydrolysate. This discovery allows the assumption that a sample with a higher degree of hydrolysis most likely also had a higher amount of small peptides.

A combination of hydrophobic properties and molecular weights estimation was figured out on the fish hydrolysate peptides with RP – HPLC. Due to the fact that for example tripeptides consisting of 3 strongly hydrophobic amino acids would come off the column earlier than hydrophilic dipeptides and vice versa made a clear size determination not possible. Nevertheless a difference between the enzymes and the hydrolysis time was once more evident. Protease M and Protease P resulted distinctive and well separated peaks, when comparing with standards, with peptides around 200 – 400Da, whereas Protamex could not obtain peaks with such a high absorbance. Nearly the same case was observed with the hydrolysis times, where the 1h variant recorded relatively low peaks in comparison to 3h and 12h hydrolysed samples.

The chromatographic results proved the preferred efficacy of Protease M and Protease P verses Protamex of producing smaller, more hydrophobic and tendentially peptides with a higher antioxidant activity. In addition, it can be stated that in most cases 1h hydrolysis correlated bigger peptides with weak bioactivity.

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