MP 179
Food Safety I

Rapid Authentication of Fish Species using Peptide Probes Isolated in the ProTrap XG

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SUMMARY

- Either 2% SDS, or 80% formic acid, provides optimal extraction of proteins from fish muscle.
- Rapid (5 min) acetone precipitation in the ProTrap XG isolates fish protein in high purity.
- Following tryptic digestion, the ProTrap XG SPE cartridge selectively isolates peptide fractions.
- Rapid MS infusion of fractionated peptides reveals an abundance of species-specific peptides.
- Protein sequence similarities across related species demands further work to identify makers.

BACKGROUND

Food adulteration is a significant problem, brining both economic and health concerns. The standard Kjeldahl assay only infers protein abundance and is easily falsified, leading the AOAC to call on more robust assays for protein content in foods. Fraudulent claims of species origin are also of concern. For example, cheaper species of fish may be unknowingly sold to the consumer. The use of mass spectrometry to identify species by peptide markers are a reliable alternative, but require lengthy sample preparation prior to analysis.

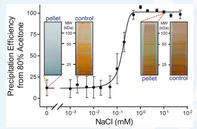


Figure 1: Acetone precipitation requires salt[2]

SDS is favored for protein extraction. Our lab has also demonstrated that high concentrations of cold formic acid are ideal for protein solubilization[1], though each system interferes with MS analysis. Acetone precipitation provides the most reliable means of recovering protein in high purity, and with high yield. The 'trick' to ensuring high protein yield through precipitation is to add a pinch of salt[2] (Figure 1). We have also proven acetone precipitation can be extremely rapid (see ThP 362 for details on kinetics).

APPROACH

- Our goal is to develop an approach to rapidly and reliably isolate peptide markers that can be used to authenticate fish species through tandem mass spectrometry.
- A secondary objective is to develop a robust assay to assess total protein content in food .
- Our strategy first involves extraction of total protein from homogenized fish muscle (Figure 2) into high concentrations of SDS or cold formic acid, followed by acetone precipitation in the ProTrap XG to recover purified protein.[3]
- The protein is resolubilized and digested with trypsin. Peptides are fractionated through the ProTrap XG SPE cartridge and analyzed by MS (direct infusion).

Figure 2: The four fish species used in this workflow.

MS Instrumentation

A low resolution, low scan speed (1 Hz) LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific) was used to perform the analysis, demonstrating that only basic MS equipment is needed with our optimized sample preparation strategy.

SAMPLE PREP: The ProTrap XG

The ProTrap XG is a disposable, two-stage filtration and extraction cartridge, designed to automate protein precipitation.

- Place fish protein extract in the ProTrap XG. Add acetone (80%) and 100 mL NaCl (Figure 3A).
- Incubate 5 min; remove plug and centrifuge 1 min to isolate pellet (3B).
- Reattach plug. Resolubilize in 8M urea for 5 min with sonication (3C).
- Add trypsin. Digest 5 min -> overnight.
- Fractionate peptides by attaching SPE cartridge to base of ProTrap XG.
- Elute peptides through 1 min spins with 400 L of 20, 30, 40, and 50% acetonitrile (Figure 3D).

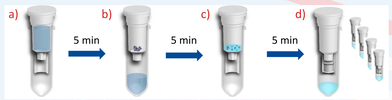
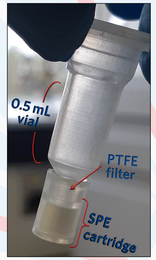


Figure 3: Workflow for precipitation (a,b), digestion (c) & separation of peptides (d) with the ProTrap XG



The ProTrap XG, with SPE cartridge attached.

RESULTS: EXTRACTION

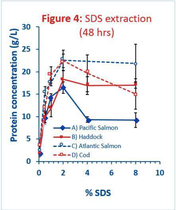


Figure 4: SDS extraction (48 hrs)

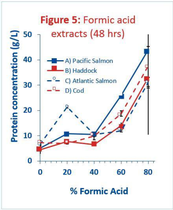


Figure 5: Formic acid extracts (48 hrs)

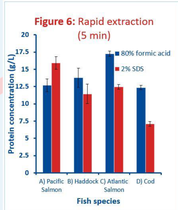


Figure 6: Rapid extraction (5 min)

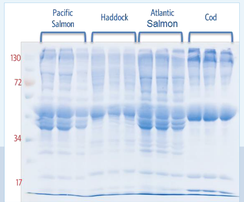


Figure 7: SDS PAGE of fish extracts (2% SDS, 48 hrs)

Rapid Extraction of Total Protein

- Exhaustive extraction (48 hrs) provides near quantitative recovery of total protein in the presence of 2% SDS (Figure 4), or 80% formic acid (Figure 5).
- With these optimal solvent systems, 60% of total protein is rapidly extracted into solution with only 5 min incubation (Figure 6).
- Proteins isolated from each solvent system will require further purification, which is accomplished via precipitation.

RESULTS: PRECIPITATION

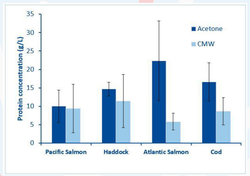


Figure 8: Protein recovery from acetone precipitation is consistently higher than precipitation via chloroform

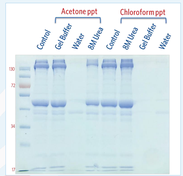


Figure 9: Resolubilization of the acetone pellet (Cod) with 8M urea (5 min)

- Acetone precipitation is the optimal strategy to purify proteins in high yields following extraction in 2% SDS (Figure 8).
- Samples are incubated at room temperature in 80% acetone, with 100 mM NaCl (5 min) to precipitate the proteins. Quantitative recovery is obtained.
- The acetone pellet is readily solubilized in 8 M urea (Figure 9), with sonication (5 min). The chloroform pellet shows lower recovery.

RESULTS: DIGESTION

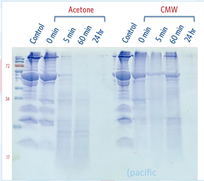


Figure 10: While 24 hour digestion with trypsin shows complete digestion of intact proteins, multiple digestion products are visible in 5 min.

Figure 11: LC/UV shows separation of peptides from digestion time course in ProTrap XG.

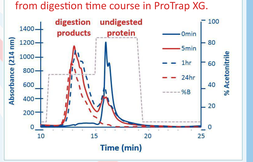


Table 1: Quantifying the % protein digested

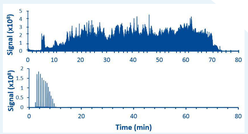
Digestion time	Total (Percent Recovery)	Peptides	% Digested (peptides/ total)
0 min	48.9	9.9	20.2
5 min	57.4	43.0	74.8
60 min	72.0	50.4	69.9
24 hr	53.9	47.2	87.6

Rapid Digestion of Total Protein

- Solvent-precipitated protein is quickly resolubilized in 8 M urea (5 min), and digested with trypsin in the ProTrap XG.
- SDS PAGE shows generation of tryptic digestion products (Figure 10).
- ~75% of protein is digested within 5 min, albeit longer digestion times (24 hrs) reveal smaller digestion products.
- The resulting peptides can be fractionated by the ProTrap XG SPE cartridge ahead of MS analysis

RESULTS: MS ANALYSIS

Figure 12: LC/MS (top) vs direct infusion (bottom) MS of an unknown sample. Data dependant analysis ID's multiple peptides



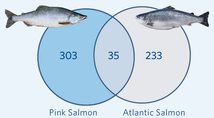


Figure 15: Venn diagram of protein ID'd from salmon

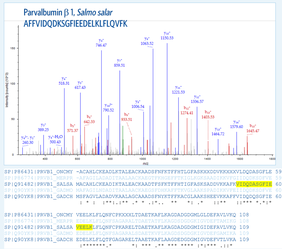


Figure 16: Peptide attributed to parvalbumin (Atlantic salmon); sequence alignment reveals this protein to be highly conserved.

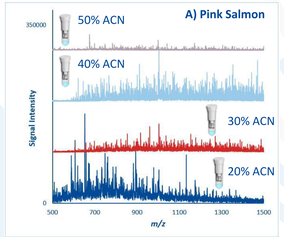


Figure 13: MS infusion of SPE fractions following ProTrap XG digestion. Most peptides elute in the 20% acetonitrile fraction.

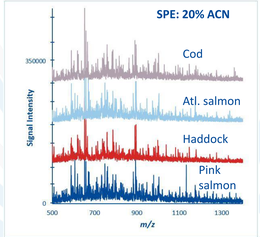


Figure 14: Comparison of peptides from MS infusion of 20% acetonitrile fraction.

Bottom-up Proteome Analysis

- Through conventional LC-MS² can be performed for detailed proteome analysis, a more rapid strategy is afforded using the ProTrap XG to fractionated tryptic peptides (Figure 12).
- Following digestion in the ProTrap XG, peptides are recovered using the integrated SPE cartridge, where buffer components are removed, and peptides are separated by successive washes with increasing ACN.
- MS infusion can be complete in under 5 min per sample, wherein the majority of peptides are observed from a 20% acetonitrile wash of the ProTrap XG SPE cartridge (Figure 13).
- Comparison of the eluted peptides from different fish species reveals several identical MS features (Figure 14), presumably from conserved proteins.
- However, uniquely identified proteins confer the identification of the fish species (Figure 15).
- It is cautioned, however, that the correct identification of a species depends significantly on the protein database. While some databases are near complete (Atlantic salmon = >86,000 entries), others are only partial (Haddock = 127 proteins).
- Further examination of identified peptides reveals that the proteins's species origin could arise from multiple different species.
- As an example, Parvalbumin 1 is abundantly observed, but is highly conserved across multiple species, including Rainbow trout, Atlantic salmon, Atlantic cod, and Haddock (Figure 16).

CONCLUSIONS, ACKNOWLEDGEMENTS, REFERENCES

Conclusions

- Successful protocol development for rapid isolation of peptides from fish muscle.
- MS analysis can distinguish different species, but also reveals the close similarity of protein sequences.
- Further analysis is required to disclose a set of distinct peptide markers for fish authentication.

Acknowledgements

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- ProTrap XG cartridges are supplied by Proteoform Scientific (www.proteoform.com)

References

1. Doucette, A.A. *et al.* "Resolubilization of Precipitated Intact Membrane Proteins with Cold Formic Acid for Analysis by Mass Spectrometry" *J. Proteome Res.* **2014**, *13*, pp 6001-6012.
2. Crowell, A.M. *et al.* "Maximizing recovery of water-soluble proteins through acetone precipitation" *Anal. Chim. Acta* **2013**, *796*, pp 48-54.
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