Enhancing the Bioactive Peptide Profile of Cheese

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Dairy-Based Bioactive Peptides

Primary Protein → Bioactive Peptides → Inactive Fragments

Hydrolysis → Hydrolysis

Properties
- Anti-oxidant
- Anti-inflammatory
- Anti-fungal
- Anti-bacterial
- Anti-hypertensive
Dairy-Based Bioactive Peptides

Primary Protein → Bioactive Peptides → Inactive Fragments

Degree of Hydrolysis Increases

Liberation
- Enzymatic hydrolysis
- Gastrointestinal digestion

Degradation
- Extensive hydrolysis
- Metabolism
Dairy Consumption and Cardiovascular Health

• Consumption of dairy products associated with reduction in:
  – Systolic and diastolic blood pressure\textsuperscript{1}
  – Cardiovascular disease and hypertension\textsuperscript{2}

• Anti-hypertensive Components in Dairy
  – Mineral content
  – Bioactive peptides \textbf{Slow accumulation during ripening}

\textsuperscript{2}Hu et al. Nutrients. 2022. 14(14) 2936
Cardiovascular Regulation

Ang I → Peptide Inhibition
Ang II → Vasoconstriction
Ang1-7 → Vasodilation

ACE1
ACE2
Knowledge Gap

• Supplementation of dairy peptides in natural cheese to directly increase bioactive properties remains unexplored

• Dairy products’ influence on ACE2 activity has been previously underreported
Peptide-Rich Dairy Ingredients

Cheese Whey → Membrane filtration → Whey Protein Phospholipid Concentrate → Alcalase enzymatic hydrolysis → Hydrolysate Solution

Dairy Protein Hydrolysate Composition (%)

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>0.90</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.32</td>
</tr>
<tr>
<td>Lactic Acid</td>
<td>1.16</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>0.48</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.53</td>
</tr>
<tr>
<td>Moisture</td>
<td>4.41</td>
</tr>
<tr>
<td><strong>Crude Protein</strong></td>
<td><strong>81.0</strong></td>
</tr>
</tbody>
</table>

(Swaminathan, 2019)
Preliminary Work

Production of Lab-Scale Cheddar Cheese with an additional Dairy Protein Hydrolysate Ingredient: Composition, Peptide Characterization, and Effects on Enzyme Inhibition

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Introduction:
- Dairy protein hydrolysates (DPH) may possess beneficial properties due to the liberation of bioactive peptides (BP) from their primary protein structure.
- Cheese products are considered a prevalent source of BP produced by proteolysis occurring during the manufacturing and ripening process.
- Dairy BP have been known to carry positive biological effects, including inhibitory action towards Angiotensin Converting Enzymes (ACE1, ACE2).
- ACE2 has been identified as the primary binding receptor for the Severe Acute Respiratory Syndrome Coronavirus-2 spike protein.
- Research lacks to investigate the effects of the direct addition of a bioactive dairy based ingredient during natural cheese manufacturing.

Objective: To produce lab-scale natural cheese enriched with a DPH powder (hydrolyzed whey protein phospholipid concentrate, 8/8% crude protein) and to investigate inhibitory effects of water soluble extracts of sample cheeses against Angiotensin Converting Enzymes.

Methods:
- 400 mL milk/vat
  - Casein: fat ratio = 0.68:1.00
- Coagulation & Cooking
- Dry Salting: 2.25% (w/v) curd mass
- 14 Day Ripening

Table 1: Composition of lab-scale Cheddar cheese after 14-day ripening period (P < 0.05)

<table>
<thead>
<tr>
<th>DPH</th>
<th>Moisture (%)</th>
<th>Fat (%)</th>
<th>Crude Protein (%)</th>
<th>Ash (%)</th>
<th>Calcium (%)</th>
<th>Sodium (%)</th>
<th>FDM (%)</th>
<th>SM</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36.85 ü</td>
<td>29.39</td>
<td>27.11 ü</td>
<td>2.11</td>
<td>32.41</td>
<td>40.87</td>
<td>5.71</td>
<td>5.15</td>
<td></td>
</tr>
<tr>
<td>1%</td>
<td>37.41 ü</td>
<td>29.50</td>
<td>27.14 ü</td>
<td>2.19</td>
<td>33.13</td>
<td>47.12</td>
<td>5.84</td>
<td>5.20</td>
<td></td>
</tr>
<tr>
<td>2%</td>
<td>36.64 ü</td>
<td>29.54</td>
<td>27.70 ü</td>
<td>2.28</td>
<td>32.08</td>
<td>40.62</td>
<td>6.23</td>
<td>5.21</td>
<td></td>
</tr>
<tr>
<td>3%</td>
<td>38.52 ü</td>
<td>27.56</td>
<td>27.51 ü</td>
<td>2.21</td>
<td>33.35</td>
<td>45.15</td>
<td>5.75</td>
<td>5.15</td>
<td></td>
</tr>
<tr>
<td>4%</td>
<td>37.80 ü</td>
<td>26.11</td>
<td>29.65 ü</td>
<td>2.22</td>
<td>31.24</td>
<td>42.82</td>
<td>5.95</td>
<td>5.21</td>
<td></td>
</tr>
<tr>
<td>5%</td>
<td>37.35 ü</td>
<td>25.46</td>
<td>30.25 ü</td>
<td>2.19</td>
<td>30.11</td>
<td>40.64</td>
<td>5.86</td>
<td>5.23</td>
<td></td>
</tr>
</tbody>
</table>

Discussion:
- Cheese composition met Cheddar standard of identity (SOI) in terms of %Moisture but not %FDM (SOI: %Moisture < 39, %FDM > 50; Table 1).
- Increase in crude protein content observed with increase of DPH enrichment levels in treated cheese samples (P<0.05; Table 1).
- Separation between level of DPH enrichment (PC1) and between control and DPH-treated samples (PC2; Figure 2).
- Increase in level of ACE2 inhibition when reacted with WSE of control versus DPH treated cheese samples (P<0.05; Figure 3).

Future Work: Alterations to lab-scale manufacturing protocols will be made in aims of meeting the full SOI of Cheddar cheese. Further peptide characterization of WSE, including peptide identification via mass spectrometry and inhibitory effects towards Angiotensin Converting Enzymes, will be investigated. In addition, bioavailability of enriched cheeses will be evaluated utilizing an in vitro gastrointestinal model.

Acknowledgments:
- Dairy Innovation Hub
- Center for Dairy Research
- Department of Food Science, UW Madison

References:

Figure 1: Lab-scale manufacturing of Cheddar cheese (a), dry salting and DPH addition (b).

Figure 2: Score plot obtained by PCA on rawness from processed WSE peptide chromatograms.

Figure 3: Inhibition of ACE2 calculated from changes in enzyme activity when reacted with WSE from control and DPH enriched cheese samples, (P < 0.05).
Hypothesis

Natural cheese manufactured with the addition of a bioactive dairy protein hydrolysate (DPH) will exhibit increased inhibitory properties towards ACE1 and ACE2 activity, thus not requiring extended ripening times to improve its bioactive properties.
Objectives

• To produce natural cheese supplemented with a bioactive DPH ingredient by incorporation during the dry-salting step of cheesemaking

• To evaluate the retention of the DPH ingredient by analyzing press whey and cheese composition

• To characterize the bioactive properties of native and bioaccessible peptide fractions by measuring their influence on ACE1 and ACE2 activity

• To characterize the production and degradation of bioavailable peptides via mass spectrometry
Cheese Manufacturing

Standard Stirred Curd Cheddar

Dry Salting Application

Bioactive Treatment*

Control  0.6% DPH  1.2% DPH  1.8% DPH  2.4% DPH  3.0% DPH  3.6% DPH

* wt/wt basis
Cheese Manufacturing
Cheese Manufacturing
Press Whey Composition

Increased DPH Addition
Press Whey Composition

Estimated: 40-46% DPH retention in curd

* Denote significant difference by treatment (P < 0.05)
## DPH Supplemented Cheese Composition

<table>
<thead>
<tr>
<th>DPH Treatment (wt/wt)</th>
<th>Moisture* (%)</th>
<th>Fat (%)</th>
<th>Protein (%N x 6.38)</th>
<th>Salt (%)</th>
<th>MNFS* (%)</th>
<th>FDM (%)</th>
<th>S/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>38.10</td>
<td>31.18bc</td>
<td>25.67ab</td>
<td>1.55b</td>
<td>55.36</td>
<td>50.37b</td>
<td>4.06b</td>
</tr>
<tr>
<td>3.6</td>
<td>37.80</td>
<td>31.89ab</td>
<td>25.32bc</td>
<td>1.68ab</td>
<td>55.48</td>
<td>51.27a</td>
<td>4.46ab</td>
</tr>
</tbody>
</table>

n = 4

* No significant difference (P > 0.05)

a-c Denotes significant difference of means within column by One-way ANOVA and Tukey HSD (P < 0.05)

Key: MNFS, moisture in non-fat solids; FDM, fat in dry-matter; S/M, salt in moisture
Assessing Bioactive Properties of Dairy Peptides

Native → Bioaccessible → Bioavailable
Fractionating Water-Soluble Peptides From Cheese

Dilute cheese sample (2 water : 1 cheese)

Homogenization and Centrifugation

Water-Soluble Extract

Ultrafiltration: 3 kDa membrane

<3 kDa Water-Soluble Peptide Extract
DPH Treatment Increase Bioactive Effects of Young Cheese

- Control Cheese
- 3.6% DPH Cheese

N = 4, mean ± SD

DPH treatment may supply initial ACE1 inhibiting peptides

* Denotes significant difference by treatment (P < 0.05)
DPH Treatment Increase Bioactive Effects of Young Cheese

**ACE2 Inhibition (%)**

- **Control Cheese**
- **3.6% DPH Cheese**

*N = 4, mean ± SD

**Denotes significant difference by treatment (P < 0.05)**

DPH treatment may supply initial ACE2 inhibiting peptides
Assessing Bioaccessibility

**in vitro static digestion**
- Oral phase → Salivary fluid
- Gastric phase → Pepsin (pH 3)
- Intestinal phase → Pancreatin & Bile (pH 7)

**Oral Phase (2 min)**

**Gastric Phase (2 hr.)**

**Intestinal Phase (2 hr.)**
In Vitro Digestion Affects ACE1 Inhibition

Control Cheese

3.6% DPH Cheese

Measured at 30d ripening

N = 3, mean ± SD

DPH treatment may supply precursor ACE1 inhibiting peptides

n.s. no significant difference (P > 0.05)
**In Vitro Digestion Increases ACE2 Inhibition**

- Digestion increases ACE2 inhibitory properties

* Denote significant difference by treatment \((P < 0.05)\)

**Results:**

- Control Cheese
- 3.6% DPH Cheese

*Measured at 30d ripening*

\[N = 3, \text{ mean } \pm \text{ SD}\]
Assessing Bioavailability of Digested Peptides

Gastrointestinal Epithelium

Lumen

Di/tripeptides and mimetics

Blood

Na⁺

H⁺

Amino Acid

Peptide

Peptid

Na⁺

ATP- ADP

K⁺

Jackman et al., 2022
Human Caco-2 Cells Differentiate to Model Enterocytes

Sinnecker, 2014
Assessing Bioavailability of Digested Peptides

Cheese digest

2-hour incubation

Sampling
Identifying Bioactive Peptides

Native

Bioaccessible

Bioavailable

Peptide Identification

LC-MS/MS
Characterizing Occurrence of Peptide

High Occurrence of Short Chain Peptides
Characterizing Occurrence of Peptide

CC: Control Cheese
TC: Treated Cheese

Low DPH Retention During Manufacturing
Characterizing Occurrence of Peptide Degradation of Polypeptides

Release of DPH Precursors

Degradation of Polypeptides

Number of Identified Peptides Sequences

CC: Control Cheese
TC: Treated Cheese

Native Bioaccessible

Peptide Length

DPH CC TC CC TC

0 100 200 300
Characterizing Occurrence of Peptide

Non-permeable Polypeptides
Identifying Bioactive Peptides

Native

Bioaccessible

Bioavailable

LC-MS/MS

Peptide Identification

Milk Bioactive Peptide Database

A Diverse Profile of ACE1 Inhibitory Peptides
A Diverse Profile of ACE1 Inhibitory Peptides

Normalized Intensity

CC: Control Cheese
TC: Treated Cheese

Low DPH Retention During Manufacturing
A Diverse Profile of ACE1 Inhibitory Peptides

CC: Control Cheese
TC: Treated Cheese

Normalized Intensity

DPH Supplied Precursors

Peptide Sequence

Native  Bioaccessible

AY  FP  LF  LL  LW  LW  LY  VP  VR  WY  YL  YY  FA  SY  YP  GAW  GTW  LGY  MAP  RYL  YGL  YLL  WYSL  VVPP
A Diverse Profile of ACE1 Inhibitory Peptides

Normalized Intensity

Peptide Sequence

Lack of ACE1 BP in Bioavailable Fraction

CC: Control Cheese
TC: Treated Cheese

CDR.wisc.edu
But How Does It Taste?
Sensory Screening

- Savory
- Acidity
- Astringency
- Bitterness

Acceptable Cheddar flavor as determined by expert screening panel after 1 and 6 month of ripening
Conclusion

• Addition and retention of supplemental dairy peptides during the manufacture of natural cheese is possible at pilot-plant scale
  – Loss of DPH treatment during mechanical pressing

• Dairy peptide supplementation in natural cheese:
  – Increases ACE1 and ACE2 inhibitory properties of young cheese
  – May supply bioaccessible precursor bioactive peptide

• DPH supplementation may not influence bioavailable peptide fraction

• Natural cheese can mask bitter flavors of dairy hydrolysate peptides
Acknowledgments

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