Bitterness in sodium caseinate hydrolysates: Role of enzyme preparation and degree of hydrolysis

Running title: Bitterness in sodium caseinate hydrolysates

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ABSTRACT

BACKGROUND
Enzymatic hydrolysis of sodium caseinate (NaCas) may lead to the development of bitterness. Careful selection of hydrolysis conditions (i.e., enzyme preparation and duration) yielding different degrees of hydrolysis (DH) may aid in the development of low bitterness.

RESULTS
Eighteen NaCas hydrolysates were generated with four enzyme preparations (Alcalase 2.4L, Prolyve 1000, FlavorPro Whey and pepsin) to different DH values. Hydrolysate bitterness score, assessed using a trained panel (10 assessors), generally increased at higher DH values for Alcalase, Prolyve and pepsin hydrolysates. However, all FlavorPro Whey hydrolysates (DH: 0.38 to 10.62%) displayed low bitterness score values (< 26.0%) comparable to intact NaCas (13.8 ± 2.0%, p > 0.05).

CONCLUSION
Enzyme preparation and DH affect the bitterness of NaCas hydrolysates. The results are relevant for the generation of NaCas hydrolysates with reduced bitterness.

Key words: casein hydrolysates; bitterness; enzymatic hydrolysis; FlavorPro Whey.
INTRODUCTION

Caseins (CNs) represent ~ 80% (w/w) of the total protein fraction of bovine milk.\textsuperscript{1} CNs may be used as an ingredient in a wide range of food products and play an important role in the nutritional and technofunctional properties of food products.\textsuperscript{2} Intact CNs may not be suitable for certain applications, such as low pH beverages, due to their relatively poor solubility. One option to improve the limited solubility of CNs, especially at their isoelectric point (pI), is to subject them to hydrolysis with food-grade proteolytic preparations.\textsuperscript{3} However, high levels of hydrolysis of the CNs may lead to bitterness.\textsuperscript{4,5}

A positive link between bitterness and the occurrence of hydrophobic amino acids (e.g., Pro residues) in peptides has been suggested in several studies.\textsuperscript{6} CNs are relatively rich in Pro, which may be contributory to CN hydrolysate bitterness. To date, there is a limited understanding on the role of hydrolytic parameters (i.e., enzyme, temperature, pH, time, substrate, enzyme concentration, etc.) which lead to the generation of bitter peptides. Specific peptidolytic/proteolytic enzyme preparations have been linked with enhanced or reduced bitterness in CN hydrolysates.\textsuperscript{7-10} In addition, the level of hydrolysis may play a role in overall hydrolysate bitterness. Contradictory reports exist on the role of DH in CN hydrolysate bitterness. Increases in perceived bitterness have been shown with increasing degree of hydrolysis (DH) for tryptic β-CN hydrolysates.\textsuperscript{10} DH values as low as 0.5% may yielded bitterness in CNs hydrolysed with trypsin or a Bacillus proteinase.\textsuperscript{8} On the other hand, a significant reduction in bitterness (28 vs. 65%) was observed following utilisation of exopeptidases, which increased the DH of a tryptic β-CN hydrolysate from 9 to 20%.\textsuperscript{7} In contrast, no correlation between the DH of commercially available sodium caseinate (NaCas) hydrolysates and bitterness was reported.\textsuperscript{11} The aim of this study was to determine
the relationship between bitterness and DH for a range of CN hydrolysates generated with four different enzyme preparations.

MATERIALS AND METHODS

Reagents

NaCas (85.92% (w/w) protein) was provided by Arrabawn Co-op Society Ltd. (Tipperary, Ireland). The food-grade proteolytic preparations, Alcalase 2.4L (2.4 Anson U g\(^{-1}\)), Novozymes A/S, Bagsvaerd, Denmark), Prolyve 1000 (2.2 Anson U g\(^{-1}\), Lyven Enzymes Industrielles, Caen, France), FlavorPro Whey (> 55 casein protease U g\(^{-1}\)) and porcine pepsin (3000 FCC U g\(^{-1}\), Biocatalysts Ltd, Cardiff, UK) were kindly provided by the suppliers. All other reagents were of analytical grade (Sigma-Aldrich, Poole, Dorset, UK).

Hydrolysate generation and degree of hydrolysis (DH) of the NaCas hydrolysates

NaCas solutions (~ 2 L) were hydrolysed at 50°C, taking 5.0 % (w/v) solutions on a protein basis for hydrolysis at pH 2.4 (pepsin) and 9.3% (w/v) on a protein basis for hydrolysis at pH 7.0 (Alcalase, Prolyve and FlavorPro Whey) employing a pH stat (718 stat Titrino, Metrohm, Herisau, Switzerland) delivering 1N HCl (pepsin) or 2N NaOH (Alcalase, Prolyve or FlavorPro Whey) as described earlier. Different enzyme to substrate ratios (E:S) and hydrolysis times were employed to generate 18 NaCas hydrolysates having specific DH values (Table 1). The liquid enzyme preparations (Alcalase and Prolyve) were used as is for hydrolysates having a DH > 4.0% or diluted in distilled water for the DH < 4.0% immediately prior to addition to the NaCas solution. Powder enzyme preparations (FlavorPro Whey and pepsin) were directly dispersed into the NaCas solution. At the end of
the reaction, thermal inactivation was carried out at pH 7.0 and 80°C for 20 min using a water bath. The DH of the NaCas hydrolysates was determined (n=3) using the TNBS method of Adler-Nissen.\textsuperscript{12}

Bitterness evaluation of the NaCas hydrolysates

The bitterness of the intact and hydrolysed NaCas was assessed using a trained sensory panel consisting of ten assessors (6 females and 4 males) which were recruited within the Department of Biological Sciences of the University of Limerick. These panellists had previously been selected from 17 original assessors, based on their ability to detect sour, sweet, salty and bitter tastes. The panel was trained over 3 sessions using standard caffeine solutions (0, 0.025, 0.050, 0.075 and 0.100 \% (w/v) as previously described.\textsuperscript{13} Subsequently, panellists were asked to perform quantitative descriptive analysis (QDA) on the bitterness of Alcalase 19.25 DH hydrolysate. Hydrolysates were presented to panellists at 0.45\% (w/v) as the mean bitterness scores at this concentration were between the minimum and maximum bitterness thresholds of the panellists, as previously described.\textsuperscript{7} Evaluation of the test samples was conducted at room temperature (21°C) in sensory evaluation booths located in a room which was lit with 18 W halogen bulbs. At each session, the assessors evaluated one sample set. Each set consisted of intact NaCas and hydrolysates which were generated with either Alcalase, Prolyve, FlavorPro Whey or pepsin to different DH values (Table 1). NaCas (obtained from the same batch) and hydrolysate samples were resuspended in mineral water (Ballygowan, Newcastle West, Co. Limerick, Ireland) at 0.45\% (w/v) protein equivalent and were allowed to equilibrate/hydrate overnight (16 h) at 4°C. These samples were then warmed up at room temperature (21°C) for 1 h prior to each tasting session. Samples were randomly letter
coded (A, B, C, etc.) and were presented to the assessors in a random order. QDA on bitterness intensity (or bitterness score) was reported as a percentage of perceived bitterness, relative to standard solutions displaying between 0 (mineral water) and 100% (0.100% (w/v) caffeine) bitterness. At each session, standards (mineral water and 0.100% (w/v) caffeine solution) were provided to the assessors followed by the test samples which were presented in a random order. Each sample was independently evaluated during three different sessions (n=3). Therefore, each panellist attended 12 different tasting sessions (four hydrolysate sets assessed three times) to evaluate all samples.

**Statistical analysis**

One-way analysis of variance (ANOVA) was performed on the mean bitterness scores. Following ANOVA, a Student Newman Keuls post-hoc test was conducted for means comparison at a significance level of \( p < 0.05 \) using SPSS (version 22, SPSS Inc., Chicago, IL, USA).

**RESULTS AND DISCUSSION**

The bitterness scores for the intact and hydrolysed NaCas samples are summarised in Table 1. As expected, the mean bitterness score for intact NaCas was low (average bitterness 13.8 \( \pm \) 2.0%). The bitterness of all FlavorPro Whey hydrolysates was similar to that of intact NaCas \( (p > 0.05, \text{Table 1}) \). At low DH values, i.e., \( \leq 3.41, 0.19 \) and 4.33% for Alcalase, Prolyve and pepsin, respectively (Table 1), hydrolysate bitterness was not significantly different \( (p > 0.05) \) from that of intact NaCas. At higher DH values, Alcalase, Prolyve and pepsin hydrolysates had higher bitterness values than NaCas \( (p < 0.05, \text{Table 1}) \). However,
across all hydrolysates, there did not appear to be a direct relationship between DH and bitterness score.

The impact of bitterness on food rejection has well been documented. Therefore, the development of protein hydrolysates with low levels of bitterness is essential for their formulation into various foods. Bitterness in CNs following enzymatic hydrolysis has been linked to the release of hydrophobic peptides. In previous studies, increased bitterness of β-CN hydrolysed with trypsin and whey protein concentrate (WPC) hydrolysed with Alcalase 2.4L, Prolyve 1000 and Corolase 7089 was reported at higher DH values. Similar results were seen herein with Alcalase, Prolyve and pepsin NaCas hydrolysates. However, the enzyme preparation used was also a determining factor in the bitterness perceived in the NaCas hydrolysates. One enzyme preparation, FlavorPro Whey, was identified herein for its ability to yield NaCas hydrolysates (DH < 11%) having mean bitterness values similar to intact NaCas (p > 0.05). FlavorPro Whey is a proteolytic preparation derived from Aspergillus spp with uncharacterised cleavage specificity, which has (according to the manufacturer, Biocatalysts) been developed for its ability to reduce whey protein hydrolysate bitterness. Apart from FitzGerald and O'Sullivan who recently reported its ability to yield low bitterness CN hydrolysates, information on its role in the generation of low bitterness food protein hydrolysates does not appear in the peer-reviewed literature. The commercial Aspergillus-derived enzyme preparation, Flavourzyme (Novo Nordisk, Bagsvaerd, Denmark), which contains both endoproteinase and exopeptidase activities, has been shown to be effective in obtaining less bitter milk protein hydrolysates compared to other enzyme preparations. The debittering ability of Flavourzyme has been linked to the presence of exopeptidase activities within this preparation. Exopeptidase activities can cleave at the C- or N-terminal side of hydrophobic amino acid residues,
thereby bringing about a reduction in bitterness.\textsuperscript{19} A possible explanation for the low bitterness of the FlavorPro Whey NaCas hydrolysates may therefore be linked to the presence of exopeptidase activities within this enzyme preparation. This hypothesis needs to be confirmed by specifically characterising the proteolytic and peptidolytic activities present within FlavorPro Whey.

\textbf{CONCLUSIONS}

FlavorPro Whey was identified for its ability to yield non-bitter NaCas hydrolysates over a range of DH values (0-11\%). The data generated herein may be employed to inform the selection of enzyme preparations and target DH values to generate hydrolysates with adequate sensory properties for future food formulations. Such hydrolysates may find application in the development of protein/peptide-fortified ready-to-drink infant formulae, fruit juices and low pH beverages.

\textbf{ACKNOWLEDGEMENTS}

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\textbf{CONFLICTS OF INTERESTS}

The authors have no conflicts of interest, financial or otherwise.

\textbf{REFERENCES}


<table>
<thead>
<tr>
<th>Set</th>
<th>Sample</th>
<th>DH†</th>
<th>E:S†</th>
<th>Hydrolysis time (min)†</th>
<th>Mean bitterness score††</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alcalase</strong></td>
<td>Intact NaCas</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.6 ± 2.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<td></td>
<td>Alcalase 0.68 DH</td>
<td>0.68 ± 0.04</td>
<td>0.004</td>
<td>2</td>
<td>12.6 ± 3.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<td>Alcalase 1.35 DH</td>
<td>1.35 ± 0.05</td>
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<td>12.8 ± 2.9&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<td>Alcalase 3.41 DH</td>
<td>3.41 ± 0.06</td>
<td>0.82</td>
<td>2</td>
<td>35.4 ± 4.3&lt;sup&gt;b,c,d,e&lt;/sup&gt;</td>
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<td>Alcalase 11.11 DH</td>
<td>11.11 ± 0.21</td>
<td>2.47</td>
<td>25</td>
<td>67.4 ± 4.9&lt;sup&gt;e,f,g&lt;/sup&gt;</td>
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<td>Alcalase 17.15 DH</td>
<td>17.15 ± 0.17</td>
<td>2.47</td>
<td>90</td>
<td>70.8 ± 3.9&lt;sup&gt;f,g&lt;/sup&gt;</td>
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<td>Alcalase 19.25 DH</td>
<td>19.25 ± 0.73</td>
<td>2.47</td>
<td>240</td>
<td>66.8 ± 4.8&lt;sup&gt;e,f,g&lt;/sup&gt;</td>
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<td><strong>Prolyve</strong></td>
<td>Intact NaCas</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.7 ± 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Prolyve 0.19 DH</td>
<td>0.19 ± 0.11</td>
<td>0.004</td>
<td>2</td>
<td>18.8 ± 3.9&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<td>Prolyve 3.68 DH</td>
<td>3.68 ± 0.06</td>
<td>0.82</td>
<td>2</td>
<td>31.5 ± 3.1&lt;sup&gt;d,e,f&lt;/sup&gt;</td>
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<td>Prolyve 9.64 DH</td>
<td>9.64 ± 0.03</td>
<td>2.47</td>
<td>29</td>
<td>79.6 ± 4.7&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>Prolyve 14.04 DH</td>
<td>14.04 ± 0.25</td>
<td>2.47</td>
<td>180</td>
<td>66.0 ± 4.8&lt;sup&gt;e,f,g&lt;/sup&gt;</td>
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<td><strong>FlavorPro Whey</strong></td>
<td>Intact NaCas</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14.4 ± 3.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<td>FlavorPro Whey 0.38 DH</td>
<td>0.38 ± 0.17</td>
<td>0.03</td>
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<td>20.0 ± 3.4&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<td>FlavorPro Whey 1.37 DH</td>
<td>1.37 ± 0.05</td>
<td>0.31</td>
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<td>22.9 ± 4.10&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<td>FlavorPro Whey 2.55 DH</td>
<td>2.55 ± 0.20</td>
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<td>20.6 ± 3.4&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<td>FlavorPro Whey 6.35 DH</td>
<td>6.35 ± 0.13</td>
<td>0.63</td>
<td>62</td>
<td>25.7 ± 2.9&lt;sup&gt;a,b,c,d&lt;/sup&gt;</td>
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<td>FlavorPro Whey 10.62 DH</td>
<td>10.62 ± 0.16</td>
<td>0.63</td>
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<td>25.7 ± 4.2&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<td><strong>Pepsin</strong></td>
<td>Intact NaCas</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>18.7± 3.7&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<td>Pepsin 0.19 DH</td>
<td>0.19 ± 0.11</td>
<td>0.025</td>
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<td>16.1± 3.3&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<td>Pepsin 1.31 DH</td>
<td>1.31 ± 0.06</td>
<td>0.25</td>
<td>2</td>
<td>21.4± 4.5&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<td>Pepsin 2.58 DH</td>
<td>2.58 ± 0.09</td>
<td>0.25</td>
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<td>24.2± 3.8&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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<td>Pepsin 4.33 DH</td>
<td>4.33 ± 0.06</td>
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<td>39.5± 6.0&lt;sup&gt;b,c,d,e&lt;/sup&gt;</td>
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<td>Pepsin 5.22 DH</td>
<td>5.22 ± 0.023</td>
<td>0.25</td>
<td>240</td>
<td>54.5± 6.7&lt;sup&gt;e,f,g&lt;/sup&gt;</td>
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</table>

†DH values and hydrolysis conditions (time and enzyme to substrate ratio (E:S)) are taken from Rajarathnam et al.†

††Each value is the mean bitterness score obtained from 10 assessors ± standard error of the mean (SEM) in triplicate (n=3). Values
with different superscript letters are significantly different ($p < 0.05$).