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Impact of enzyme preparation and degree of hydrolysis on peptide profile and nitrogen solubility of sodium caseinate hydrolysates

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Abstract

The solubility of casein ingredients is limited under certain pH conditions. Therefore, sodium caseinate (NaCN) was hydrolysed at laboratory scale with four enzyme preparations (Alcalase, Prolyve, FlavorPro Whey and pepsin) yielding hydrolysates having degree of hydrolysis (DH) values between 0.19 ± 0.11 to 19.25 ± 0.73%. The nitrogen solubility index (NSI) over the pH range 2.0-8.0 was affected by (a) the specificity of the enzyme preparation, (b) the DH and (c) the presence of unhydrolysed NaCN within the hydrolysate. The trends observed at laboratory scale (< 5 L) were also seen with the semi-pilot scale (300 L) hydrolysates generated with Alcalase and FlavorPro Whey. Removal of unhydrolysed NaCN from the FlavorPro Whey hydrolysate using a 5 kDa ultrafiltration (UF) membrane increased NSI. This study has highlighted the importance of carefully selecting the proteolytic preparation along with the DH to design casein-based ingredients with enhanced technofunctional properties.

Key words: nitrogen solubility; casein; peptides; technofunctional properties; degree of hydrolysis
Introduction

Milk proteins are employed in the formulation of numerous food products for their nutritional, sensory and technofunctional properties. Caseins (CN), which represent the most abundant protein from bovine milk (80% (w/w)), possess numerous technofunctional properties such as emulsifying, gelling, water and lipid binding as well as texturizing properties (Cayot & Lorient, 1998; Carr & Golding, 2016). The main limitation of sodium caseinate (NaCN), a commercially available CN-rich ingredient, for wide ranging application as a technofunctional food ingredient is related to low solubility at its isoelectric point (pI = 4.6; Broyard & Gaucheron, 2015). High solubility of CN-based ingredients is often necessary to ensure their adequate incorporation into foods during formulation and further processing. In addition, solubility may be considered as a prerequisite for many functional properties such as foaming, emulsification and clarity. It has been suggested that the technofunctional applications of NaCN may be improved if solubility around the pI is increased (Panyam & Kilara, 1996). In this context, the utilisation of proteolytic enzymes has been proposed as a means to enhance NaCN technofunctional properties (Chobert et al., 1988a; Chobert et al., 1988b; de Castro et al., 2015; Broyard & Gaucheron, 2015). In addition to added technofunctional benefits, milk protein hydrolysis has also been shown to release bioactive peptides which may be relevant to human health (Nongonierma & FitzGerald, 2015; Hsieh et al., 2015; Nongonierma et al., 2016). A wide range of food-grade proteolytic enzyme preparations have been described for the hydrolysis of milk protein substrates with the view of generating both bio- and technofunctional hydrolysates (Nongonierma & FitzGerald, 2011). The extent of CN hydrolysis may be
characterised by several physicochemical parameters, of which the degree of hydrolysis (DH) is
a common approach employed to calculate the percentage of peptide bonds cleaved during
hydrolytic reactions (Adler-Nissen, 1986; Spellman et al., 2003). It is thought that the increased
solubility on protein hydrolysis is linked to the generation of lower molecular mass and
hydrophilic peptides as well as the increased number of ionised groups (-NH₄⁺ and -COO⁻),
which results in a higher extent of interaction with water molecules (Turgeon et al., 1992;
Nielsen, 1997; de Castro et al., 2015). It could therefore be postulated that at higher DH values,
CN hydrolysates would be more soluble. Hydrolysis of NaCN using different proteolytic
preparations has been shown to enhance hydrolysate solubility over a wide range of pH values.
Hydrolysis of CN using trypsin (Chobert et al., 1988a) and Staphylococcus aureus V8 proteinase
(Chobert et al., 1988b) resulted in increased solubility near the pI. The solubility of Bacillus
proteinase (Protamex™, Novozymes, Bagsvaerd, Denmark) generated NaCN hydrolysates
increased with increasing DH, particularly between pH 4.0-6.0 (Slattery & FitzGerald, 1998).
Luo et al. (2014) hydrolysed NaCN with three proteolytic preparations (papain, trypsin and
pancreatin) to generate hydrolysates having a wide range of DH values. They demonstrated that
higher extents of CN hydrolysis yielded samples where solubility was pH-independent within the
pH range 3.0-9.0. Only a limited number of studies have attempted to establish a link between
increased solubility of CN hydrolysates and their DH. In addition, the role of the enzyme
specificity in modifying the solubility is still not fully understood.
Depending on the enzyme activities present in the proteolytic preparation and the extent of
hydrolysis achieved, a wide variety of peptide sequences may be generated which lead to
different physicochemical and technofunctional properties. However, it would appear that there
are only a limited number of studies reported in the literature in which comparative nitrogen
solubility and physicochemical characterisation studies have been performed on hydrolysates
generated from the same CN substrate. To our knowledge, no such studies have been conducted
using the enzyme preparations selected herein. In addition, most of the information available in
the literature appears to have been obtained at a laboratory scale. Therefore, the first objective of
this study was to generate NaCN hydrolysates with different DH values using commercially
available food-grade proteinase preparations from bacterial (Alcalase 2.4L and Prolyve 1000
from *Bacillus licheniformis*), fungal (FlavorPro Whey from *Aspergillus* spp) and mammalian
(pepsin from porcine gastric mucosa) sources. The second objective was to attempt to link the
specificity of the enzymes used and the physicochemical characteristics (DH, peptide profile and
molecular mass distribution) of the hydrolysates to their solubility characteristics. As a third
objective, selected hydrolysates were generated at semi-pilot scale to verify the relevance of the
findings herein and to ensure hydrolysis process transferability to an industrially relevant larger
scale.

**Materials and Methods**

**Reagents**

NaCN was provided by Arrabawn Co-op Society Ltd. (Nenagh, Ireland). The protein content of
the NaCN substrate was 85.92% (w/w) as determined using the macro-Kjeldahl procedure (IDF,
1993). The food-grade proteolytic preparations, Alcalase 2.4L (2.4 Anson units g⁻¹) from
Novozyme A/S (Bagsvaerd, Denmark), Prolyve 1000 (≥ 3000 U g⁻¹ protein) from Lyven
Enzymes Industrielles (Caen, France), FlavorPro Whey (> 55 Casein Protease units g$^{-1}$ protein) and porcine pepsin (3000 pepsin units g$^{-1}$ protein) from Biocatalysts Ltd (Cefn Coed, Wales, UK) were kindly provided by the suppliers. Trinitrobenzene sulphonic acid (TNBS, Pierce Biotechnology, Rockford, IL, USA) was purchased from Medical Supply (Dublin, Ireland). Trifluoroacetic acid (TFA), high pressure liquid chromatography (HPLC) grade acetonitrile, HPLC grade water, sodium dodecyl sulphate (SDS), Leu, sodium phosphate monobasic, sodium phosphate dibasic, concentrated H$_2$SO$_4$ (low in nitrogen), HCl and NaOH solutions used in this study were from Sigma-Aldrich (Poole, Dorset, UK). Kjeldahl catalyst tablets were purchased from BDH (Leicestershire, England).

**Generation of laboratory scale NaCN hydrolysates**

NaCN solutions (~ 5 L NaCN at 5.0 and 9.3% (w/v) on a protein basis for hydrolysis at pH 2.4 and 7.0, respectively) were prepared at 50$^\circ$C using an overhead stirrer (Heidolph Instruments, Schwabach, Germany). Hydrolysis was carried out at constant pH, i.e., 2.4 (pepsin) or 7.0 (Alcalase, Prolyve and FlavorPro Whey) using a pH stat (718 stat Titrino, Metrohm, Herisau, Switzerland) delivering 1N HCl or 2N NaOH as previously described (Phelan et al., 2010). For the same enzyme preparation, different enzyme to substrate ratios (E:S) and/or hydrolysis durations were used in order to generate hydrolysates with a wide range of DH values. For each enzyme, the incubation times required to reach specific DH values were previously determined by monitoring HCl or NaOH uptake over 4 h hydrolysis of the NaCN solution. The E:S ratio used and the DH values achieved for specific incubation times are summarised in Supplementary Table S1. The enzymatic reaction was terminated by thermal treatment in a water bath at 80$^\circ$C,
20 min for the neutral pH hydrolysates (Alcalase, Prolyve and FlavorPro Whey) and by pH adjustment to 7.0 followed by thermal treatment (80°C, 20 min) for the peptic hydrolysates. Samples were stored at -20°C prior to subsequent analysis.

**Generation and processing of the semi-pilot scale hydrolysates**

Two hydrolysates were generated at semi-pilot scale with Alcalase and FlavorPro Whey. A 300 L NaCN solution at 9.3% (w/v) on a protein basis, pH 7.0 was prepared by adjusting the pH of acid CN with 30% (w/v) NaOH at 50°C. The proteolytic preparation was then added at an E:S of 2.47% (v/w) for Alcalase and 0.63% (w/w) for FlavorPro Whey. Hydrolysis was carried out for 4 h at 50°C, pH 7.0. The pH was maintained constant throughout the reaction with the addition of 30% (w/v) NaOH. Mixing was performed using a Silverson high shear mixer (Silverson Machines Ltd, Buckinghamshire, UK). The enzyme was inactivated by bringing the solution to 80°C while circulating hot water in the outer jacket of the reactor and maintaining this temperature for 20 min.

In order to remove large molecular mass proteins and peptides, the hydrolysates were ultrafiltered (UF) with a DSS MemProc membrane filtration unit fitted with a DSS GR70PE membrane (Alpha-laval AB, Lund, Sweden) with a nominal molecular mass cut-off of 5 kDa. UF was performed at 50°C with an inlet pressure of 6-8 bar. Spray-drying of crude hydrolysates and their associated 5 kDa permeate and retentate was performed using a Niro Minor Sprayer (GEA Process Engineering Limited, Cheshire, UK) with two fluid atomisation nozzles using an atomising air pressure of 2 bar and inlet and outlet temperatures of 200 and 80°C, respectively.
DH of the NaCN hydrolysates

In order to determine appropriate sampling times to reach target DH values, the DH was estimated on the basis of NaOH consumption during the hydrolysis reaction as described by Adler-Nissen (1986) using Equation 1:

\[
DH = 100 \times B \times N_b \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{h_{tot}} \quad \text{Equation 1}
\]

With B, the base (or acid) consumption (mL); \(N_b\), the base (or acid) normality; \(\alpha\), the average degree of dissociation of the \(\alpha\)-NH\(_2\) groups; MP, the mass of protein hydrolysed (g) and \(h_{tot}\) the total number of peptide bonds in the protein substrate (meq g\(^{-1}\) protein; \(h_{tot} = 8.2\) meq g\(^{-1}\) for CN).

For all hydrolysates (laboratory and semi-pilot scale), the DH was subsequently measured in triplicate using the TNBS method (Adler-Nissen, 1986) as described by Spellman et al. (2003). Absorbance values were measured at 340 nm (Cary Bio 100 UV/visible spectrophotometer, Varian, Walnut Creek, CA, USA). The DH was calculated according to Equation 2:

\[
DH = 100 \times \frac{(AN_2-AN_1)}{Npb} \quad \text{Equation 2}
\]

With \(AN_1\), the amino nitrogen content of the unhydrolysed CN (mg g\(^{-1}\) protein); \(AN_2\), the amino nitrogen content of the hydrolysate (mg g\(^{-1}\) protein) and \(Npb\), the nitrogen content of the peptide bonds in the CN substrate (112.1 mg g\(^{-1}\) protein for CN).

Peptide profile and molecular mass distribution of the NaCN hydrolysates

Reversed phase (RP)- and gel permeation (GP)-HPLC of the unhydrolysed and hydrolysed NaCN samples were performed as previously described (Spellman et al., 2009).
Determination of nitrogen solubility indices (NSIs)

Nitrogen solubility analysis was performed between pH 2.0 and 8.0, in duplicate, on 4% (w/v) samples of the NaCN hydrolysates as described previously (Flanagan & FitzGerald, 2002a). For each enzyme preparation, selected hydrolysate samples were chosen to cover a wide range of DH values. The overall variability around the mean NSI value was < 1.0%.

Statistical analysis

The DH values (n=3) were analysed using a one way ANOVA followed by a Student Newman Keuls post hoc test at a significance of 5% using SPSS (version 22, SPSS Inc., Chicago, IL, USA).

Results and Discussion

DH values of the NaCN hydrolysates

For all hydrolysates, the DH estimated by the consumption of base (or acid, Supplementary Table S1) and determined by the TNBS method (Table 1) were of the same order. For the different hydrolysis reactions, it is seen that a wide range of DH values were obtained by varying the E:S ratio and/or the hydrolysis time. The lowest DH value (0.19 ± 0.11%) was obtained on incubation with pepsin or Prolyve and the highest (19.25 ± 0.73%) with Alcalase.

The DH values of the Alcalase and FlavorPro Whey semi-pilot scale hydrolysates were 19.09 ± 1.27 and 13.49 ± 0.27%, respectively (Table 1).
Alcalase and Prolyve are *Bacillus licheniformis* preparations. Both contain subtilisin Carlsberg, an alkaline serine proteinase which has broad specificity but preferentially cleaves at the carboxyl side of hydrophobic amino acid residues (Svendsen & Breddam, 1992). This broad specificity may explain the high DH values associated with the hydrolysates generated with both of these enzyme preparations. Furthermore, Alcalase contains a higher overall proteolytic activity (on a unit weight basis) than Prolyve as well as a glutamyl endopeptidase activity which is not present in Prolyve (Svendsen & Breddam, 1992; Spellman *et al.*, 2005). This may explain why the DH of the Alcalase hydrolysate was higher than that of the Prolyve hydrolysate (Table 1). The lower DH values achieved with Prolyve as opposed to Alcalase agree with the DH values obtained during whey protein concentrate hydrolysis with these enzymes in another study (Spellman *et al.*, 2009). FlavorPro Whey is a fungal (*Aspergillus* spp) proteolytic preparation with unknown specificity. Pepsin, an aspartic protease which operates at low pH, preferentially cleaves after hydrophobic amino acid residues such as Phe and Leu (Nongonierma & FitzGerald, 2011). The DH values obtained with pepsin were the lowest for the four enzyme preparations studied, which may be linked to its defined specificity and with the relatively low E:S (≤ 0.25% (w/w)) used during the hydrolysis reaction. This result is in agreement with earlier studies reporting a DH of 5% for a peptic β-CN hydrolysate (Monogioudi *et al.*, 2011).

**Peptide profile and molecular mass distribution of the NaCN hydrolysates**

The RP-HPLC profile of the unhydrolysed NaCN is illustrated in Figure 1. The profile of intact NaCN displays two large peaks eluting at 52.4 and 53.4 min which may represent αs1- and β-CN, respectively (Baxter *et al.*, 2007; Bonizzi *et al.*, 2009).
The RP-HPLC profiles of the different hydrolysates generated at different DH values with the four proteolytic preparations are also shown in Figure 1. Over the course of the hydrolysis reaction, degradation of intact CNs can be seen, resulting in the appearance of peptide peaks. As expected, when the DH increases, peptide peaks become more abundant and/or diverse. The peptide profiles of hydrolysates generated with different proteolytic preparation differed. This was related to differences in hydrolytic specificity within the proteolytic preparations.

The molecular mass distribution profiles of the laboratory scale NaCN hydrolysates are summarised in Table 1. The extent of NaCN degradation varied depending on the enzyme preparation used during hydrolysis. As expected, for hydrolysates obtained with the same enzyme, the extent of NaCN (fraction > 10 kDa) degradation increased with increasing DH values. In line with the low DH values obtained on hydrolysis with pepsin, these hydrolysates contained a large proportion of unhydrolysed CN (> 25% material > 10 kDa). In the case of the hydrolysates with DH values > 10% (generated with Alcalase, Prolyve and FlavorPro Whey), more than 90% of the hydrolysate components had a molecular mass < 5 kDa (Table 1).

The RP-HPLC profiles of the semi-pilot scale hydrolysates obtained with Alcalase and FlavorPro Whey are displayed in Figures 2a and 2d. Differences in peptide profiles were seen between the NaCN Alcalase semi-pilot scale hydrolysate and its 5 kDa retentate and permeate (Figures 2a, 2b and 2c). Peptide peaks eluting at similar retention times were seen in all three samples, however, peak intensities varied between the three samples. The peptide profiles of the NaCN FlavorPro Whey semi-pilot scale hydrolysate and its 5 kDa retentate and permeate (Figures 2d, 2e and 2f) were different from each other. The profile of the hydrolysate and its associated 5 kDa retentate (Figures 2d and 2e) contained peaks corresponding to unhydrolysed CN (eluting around 53 min),
which were absent from the 5 kDa permeate (Figures 2f). In addition, peptide peaks in the 20-34 min region of the hydrolysate were increased in abundance in the 5 kDa permeate.

The semi-pilot scale Alcalase hydrolysate contained > 99% peptides < 5 kDa (Table 1). Therefore, as expected, processing of the hydrolysate through a 5 kDa UF membrane had little or no effect on further fractionation. This is in agreement with the RP-HPLC profiles showing similar peptide peaks in this hydrolysate and its associated UF fractions, eventhough differences in peak intensities could be seen (Figures 2a, 2b and 2c). Approximately 25% of the components in the FlavorPro Whey semi-pilot scale hydrolysate was > 10 kDa (Table 1). However, the UF step completely removed this large molecular mass material, effectively increasing the proportion of material < 5 kDa to > 99% in the permeate (Table 1).

**NSI of the NaCN hydrolysates**

The NSI of selected NaCN hydrolysates generated with Alcalase, Prolyve, FlavorPro Whey and pepsin at laboratory scale is illustrated in Figure 3. The different hydrolysates are labelled according to their DH values as determined using the TNBS method. As expected, the NSI of the unhydrolysed NaCN was minimal at the pI region of CN (pH 4.0-5.0).

For the hydrolysates, differences in NSI were seen depending on the enzyme preparation and the DH. Pepsin hydrolysates generally had a relatively low solubility (Figure 3d) which may be linked to the high proportion (> 25%) of unhydrolysed NaCN within samples (Table 1). The 10.48 and 11.11% DH Alcalase (Figure 3a) and the 9.64% DH Prolyve (Figure 3b) hydrolysates had similar NSI profiles. However, the FlavorPro Whey 10.62% DH profile (Figure 3c) showed a comparably lower solubility (60-80%) throughout the entire pH range studied (Figure 3c).
Approximately 100% solubility was achieved in the case of the Alcalase 19.25% DH (Figure 3a) and the Prolyve 14.04% DH (Figure 3b) hydrolysates across the pH range tested.

For hydrolysates with DH values < 9%, the solubility increased as DH increased between pH 2.0 to 5.0 (Figure 3). Compared with unhydrolysed NaCN, a decrease in solubility at pH 6.0 and 7.0 at low DH was observed for the 3.41% DH Alcalase (Figure 3a), the 3.68% DH Prolyve (Figure 3b), the 6.35 and 6.98% DH FlavorPro Whey (Figure 3c), and the 2.58 and 4.33% DH pepsin hydrolysates (Figure 3d). A decrease in solubility at pH 6.0 and 7.0 at low DH has previously been reported with CN hydrolysates (Flanagan & FitzGerald, 2002b; Slattery & FitzGerald, 1998; Chobert et al., 1988a; Chobert et al., 1988b). At low DH values, some of the peptides released may have higher pIs, which may result in a decrease in solubility around neutral pH. The loss in solubility may also arise from a pH-dependent reduction in ionisable groups and an increase in hydrophobic residue exposure leading to decreased electrostatic repulsion and increased hydrophobic interactions between peptides at higher pH values. Such modifications in the physicochemical interactions between peptides have previously been suggested to explain the solubility decrease of soy protein hydrolysates at pH values > 6.0 (Walsh et al., 2003).

For all enzyme preparations, the solubility of the hydrolysates around the pI increased as the extent of hydrolysis (DH) increased (Figure 3). Similar results have been reported earlier with NaCN hydrolysates also showing modifications in the NSI around the pI of CN, which was enzyme and DH dependant (Luo et al., 2014). Enhancement in solubility of CN hydrolysates in the pI region of CN has previously been reported following hydrolysis with trypsin (Chobert et al., 1988a) and S. aureus V8 proteinase (Chobert et al., 1988b). Increasing the extent of hydrolysis of NaCN using Protamex™ has also been shown to improve the solubility of the
hydrolysates at pH 4.6 (Flanagan & FitzGerald, 2002b; Slattery & FitzGerald, 1998). This increased solubility around the pI has been mainly attributed to a reduction in molecular mass, generation of hydrophilic peptides and an increase in the number of ionisable groups of the peptides generated following enzymatic hydrolysis (Panyam & Kilara, 1996; Hayakawa & Nakai, 1985; Nielsen, 1997; Luo et al., 2014).

Two hydrolysates (obtained with Alcalase and FlavorPro Whey) were produced at semi-pilot scale (300 L) to verify that the trends observed with the laboratory scale (< 5L) hydrolysates were also seen at a larger scale. This scale-up step was considered relevant to an industrial context as ultimately the results herein may find application in the generation of milk protein-based ingredients with enhanced solubility profiles. The NSI profile obtained with the 19.09% DH Alcalase hydrolysate generated at semi-pilot scale and its 5 kDa permeate (Figure 4a) showed 100% solubility over the entire pH range tested. As expected, there were little differences between the Alcalase semi-pilot scale hydrolysate and its associated 5 kDa permeate as the crude hydrolysate did not contain material > 5 kDa (Table 1). The 13.49% DH FlavorPro Whey hydrolysate generated at semi-pilot scale (Figure 4b) had a solubility between 60-80% across the pH range tested. However, the 5 kDa permeate of the semi-pilot scale hydrolysate (Figure 4b) displayed 100% solubility over the entire pH range. This superior solubility of the 5 kDa permeate over that of the FlavorPro Whey hydrolysate may be linked to the higher proportion of low molecular mass compounds (< 5 kDa) and the absence of intact NaCN in the permeate (Table 1).

Membrane processing may be used to remove high molecular mass peptides from protein hydrolysates. While data is available for other milk protein substrates, very little work appears to
have been conducted on UF fractions of NaCN hydrolysates and their nitrogen solubility. For example, in the case of whey protein hydrolysates, it was shown that UF permeates had a higher solubility than the whey protein isolate hydrolysates when generated with Alcalase, trypsin and chymotrypsin (Mutilangi et al., 1996).

**Conclusion**

Enzymatic hydrolysis of NaCN led to significant changes in the nitrogen solubility and physicochemical properties of both laboratory (< 5 L) and semi-pilot scale (300 L) generated hydrolysates. Based on the results obtained in this study, it is evident that these changes depended upon the specificity of the proteinase preparations used. In general, nitrogen solubility in the pI region (pH 4.0-5.0) increased with increasing DH, with the extent depending on the proteolytic preparation used. This study also clearly illustrated the role of UF processing in altering the solubility profiles and the physicochemical properties of those hydrolysates containing large molecular mass components.

The hydrolysates and their UF fractions generated within this study may have potential application as ingredients in formulated food products. They may also be of use for nitrogen fortification of low pH beverages where good solubility of hydrolysates is a prerequisite. As bitterness defects may be an issue with CN hydrolysates, future work may involve assessment of the sensory characteristics of the hydrolysates displaying the most interesting solubility profiles herein. To our knowledge, this the first report outlining the comparable NSI and physicochemical properties of NaCN hydrolysates generated with Alcalase, Prolyve, FlavorPro Whey and pepsin.
Acknowledgements

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References


Table and figure captions

Table 1. Molecular mass distribution profiles and degree of hydrolysis (DH, determined by the trinitrobenzene sulphonic acid (TNBS) method) of the sodium caseinate (NaCN) hydrolysates generated at laboratory and semi-pilot scale, and of the associated 5 kDa ultrafiltration (UF) permeate of the semi-pilot scale hydrolysates.

Figure 1. Reverse-phase high-performance liquid chromatography (RP-HPLC) profiles of laboratory scale sodium caseinate (NaCN) hydrolysates at different degree of hydrolysis (DH) values. (a) Alcalase, (b) Prolyve, (c) FlavorPro Whey and (d) pepsin hydrolysates.

Figure 2. Reverse-phase high-performance liquid chromatography (RP-HPLC) profiles of (a) Alcalase- 19.09% degree of hydrolysis (DH) and (d) FlavorPro Whey- 13.49% DH generated semi-pilot scale sodium caseinate (NaCN) hydrolysates, (b and e) their 5 kDa retentates and (c and f) their 5 kDa permeates, respectively.

Figure 3. Nitrogen solubility profiles of (a) Alcalase, (b) Prolyve, (c) FlavorPro Whey and (d) pepsin sodium caseinate (NaCN) hydrolysates generated at laboratory scale. Data points plotted are means of independent duplicate determinations. Hydrolysates are labelled according to their degree of hydrolysis (DH) estimated using the trinitrobenzene sulphonic acid (TNBS) method.

Figure 4. Effect of pH on the nitrogen solubility of semi-pilot scale (a) Alcalase-generated hydrolysat e (19.09% degree of hydrolysis (DH)) and (b) FlavorPro Whey generated hydrolysate (13.49% DH) of sodium caseinate (NaCN) and their associated 5 kDa permeates. Data points plotted are means of independent duplicate determinations, overall variability < 1.0%.
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<tr>
<th>Enzyme</th>
<th>Sample</th>
<th>Degree of hydrolysis (% DH)</th>
<th>Molecular mass distribution (% area)</th>
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<td>13.27 ± 0.13&lt;sup&gt;l,m&lt;/sup&gt;</td>
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<td>17.15 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>19.25 ± 0.73&lt;sup&gt;p&lt;/sup&gt;</td>
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<td>laboratory scale hydrolysate</td>
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<tr>
<td></td>
<td></td>
<td>1.37 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.55 ± 0.20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>64.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.35 ± 0.13&lt;sup&gt;g&lt;/sup&gt;</td>
<td>17.32</td>
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<td>6.98 ± 0.16&lt;sup&gt;h&lt;/sup&gt;</td>
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<td>9.63 ± 0.16&lt;sup&gt;i&lt;/sup&gt;</td>
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<td>10.62 ± 0.16&lt;sup&gt;ijk&lt;/sup&gt;</td>
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1 Each value is the mean DH ± SD (n=3). Values with different superscript letters are significantly different (p < 0.05).
Figure 1

Detector response at 214 nm

Retention time (min)
Figure 2
Figure 4

(a) Nitrogen solubility (%) versus pH for Semi-pilot scale hydrolysate and 5 kDa permeate.

(b) Nitrogen solubility (%) versus pH for Semi-pilot scale hydrolysate and 5 kDa permeate.
Supplementary Table S1. Enzyme to substrate ratio (E:S) used to hydrolyse sodium caseinate (NaCN) with Alcalase, Prolyve, FlavorPro Whey and pepsin. The incubation times used and degree of hydrolysis (DH) values achieved, as estimated from NaOH and HCl uptake during the hydrolysis reaction, are also shown.

<table>
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<tr>
<th>Enzyme</th>
<th>E:S (%) (w/w) or (v/w)</th>
<th>Incubation time (min)</th>
<th>Sample DH (%)</th>
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<td>-</td>
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1The enzyme to substrate ratio (E:S) is expressed as % (v/w) for Alcalase and Prolyve, and as % (w/w) for FlavorPro Whey and pepsin on a protein basis of sodium caseinate (NaCN).