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# Total Solids Content and Degree of Hydrolysis Influence Proteolytic Inactivation Kinetics Following Whey Protein Hydrolysate Manufacture

Celia Conesa and Richard J. FitzGerald*

Department of Life Sciences, University of Limerick, Limerick, Ireland

**ABSTRACT:** The kinetics and thermodynamics of the thermal inactivation of Corolase PP in two different whey protein concentrate (WPC) hydrolysates with degree of hydrolysis (DH) values of ~10 and 21%, and at different total solids (TS) levels (from 5 to 30% w/v), were studied. Inactivation studies were performed in the temperature range from 60 to 75 °C, and residual enzyme activity was quantified using the azocasein assay. The inactivation kinetics followed a first-order model. Analysis of the activation energy, thermodynamic parameters, and $D$ and $z$ values, demonstrated that the inactivation of Corolase PP was dependent on solution TS. The intestinal enzyme preparation was more heat sensitive at low TS. Moreover, it was also found that the enzyme was more heat sensitive in solutions at higher DH.

**KEYWORDS:** *thermal inactivation kinetics, Corolase PP, whey protein hydrolysate, total solids, degree of hydrolysis*

## INTRODUCTION

Protein ingredients are used for a variety of techno- and biofunctional applications in the food industry. Modification of food proteins based on enzymatic hydrolysis can be a valuable bioprocess to enhance the functional properties of the original protein without prejudicing its nutritive value.[1]

Protein hydrolysates can be produced from precursor proteins in different ways including (a) enzymatic hydrolysis by digestive enzymes, (b) fermentation with proteolytic starter cultures, (c) proteolysis by enzymes derived from mammals, microorganisms, or plants, or (d) combinations of the above. Production of hydrolysates by the action of endopeptidases and exopeptidases is considered the most effective way to obtain protein hydrolysates with defined characteristics.[2] This approach exploits the unique cleavage specificity of the enzyme(s) used and bypasses issues with the use of concentrated acid or alkaline or with residual solvents/toxic chemicals associated with the organic synthesis of peptides.[3,4]

Some of the beneficial technofunctional properties of enzymatic hydrolysis of proteins include modification to the solubility, gelation, emulsification, foaming, and bitterness.[5−7] Predigestion of proteins reduces their allergenic potential and may enhance their bioavailability. Therefore, the use of hydrolysates is important in the preparation of diets suitable for individuals with specific needs, for hypoallergenic infant formulas, and for dietetic foods and sport drinks.[1,2] Moreover, it has also been increasingly recognized that dietary proteins exert many functions in vivo due to the presence of bioactive peptides (BAPs) within their sequences. These food-derived BAPs may perform beneficial physiological effects and therefore have the potential to enhance health.[8] These peptides are inactive within the sequence of the parent protein but can be released on hydrolysis with specific enzymes both in vivo and in vitro.[9] Many biological functions have been attributed to these food-derived peptides including antimicrobial, antioxidant, antithrombotic, antihypertensive, immunomodulatory, opioid, and antitumor activities.[8,10−12]

Although hydrolysates can be obtained from a wide range of food proteins such as egg, fish, meat, cereals, and soybeans, milk protein is currently the most widely studied source of peptides.[3,13] The conventional industrial approach to making protein hydrolysates involves incubation of the protein with an appropriate proteolytic enzyme preparation. The characteristics of the final hydrolysate depends on factors such as the protein substrate, proteolytic enzyme, amount of total solids (TS), enzyme to substrate ratio (E:S), pH, temperature, hydrolysis time, and degree of hydrolysis (DH) achieved.[14,15] From a practical perspective, when the desired characteristics of the hydrolysate have been reached, the enzyme used in the reaction needs to be inactivated. This step prevents further hydrolysis of the hydrolysate itself or of other proteins if the hydrolysate is subsequently incorporated as an ingredient with other intact protein components. The most common approach for enzyme inactivation is by heat treatment, leading to denaturation of the enzyme. Some information exists in the literature in relation to the thermal inactivation of food-processing enzymes such as pectinases,[16] polyphenol oxidases,[17,18] and peroxidases,[19,20] among others. However, little or no detailed information appears to exist in the peer-reviewed scientific literature in respect to the thermal inactivation kinetics of proteolytic preparations used in the generation of food protein hydrolysates. It is known that different heat treatments may affect the nutritional, technofunctional, and biofunctional properties of food protein hydrolysate ingredients.[21,22] Therefore, detailed studies with respect to optimization of thermal treatments for enzyme inactivation have relevance with respect to hydrolysate properties per se and for the economics of hydrolysate manufacture. The objective of this work was to determine the effect, if any, of solution TS content and DH on the thermal inactivation kinetics of a mammalian

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intestinal enzyme preparation (Corolase PP) during the generation of whey protein concentrate (WPC) hydrolysates.

## MATERIALS AND METHODS

**Samples and Reagents.** Whey protein concentrate (WPC80, 80.3% protein content), manufactured from sweet whey, was obtained from Carbery Milk Products (Ballineen, Ireland). Corolase PP was kindly supplied by AB Enzymes (Darmstadt, Germany). Azocasein, trichloroacetic acid (TCA), sodium phosphate monobasic, sodium phosphate dibasic, and trifluoroacetic acid (TFA) were from Sigma-Aldrich (Dublin, Ireland). HPLC grade water, acetonitrile (ACN), and 0.2 $\mu$m PTFE filters were from VWR (Dublin, Ireland). All other reagents were of analytical grade, unless otherwise stated.

**Enzymatic Hydrolysis of WPC.** Hydrolysis was performed essentially according to the method of Spellman et al.[15] with minor modifications. A 30% (w/v) aqueous solution of WPC was incubated with Corolase PP at an enzyme to substrate ratio of 0.3% (w/w), on a protein basis, at 50 °C at pH 7.0. After 4 h of incubation, the hydrolysate sample was heated to 80 °C for 20 min to inactivate the enzyme. Aliquots of the heat-inactivated hydrolysate were then diluted in distilled water to obtain solutions at the following TS concentration: 30, 20, 10, and 5% (w/v). Samples of this first hydrolysate were stored at −20 °C until required.

**Further Enzymatic Hydrolysis of the 30% TS Hydrolysate.** An aliquot (175 mL) of the heat-inactivated 30% TS hydrolysate was equilibrated at 50 °C. Sodium azide was added at 0.02% (w/v) to prevent microbial growth, and the pH was readjusted to 7.0. Corolase PP was added at an enzyme to substrate ratio of 0.3% (w/w), as previously. After 6 h of incubation at pH 7.0, the enzyme was heat inactivated as described above. This new hydrolysate was diluted in distilled water to obtain solutions at 20, 10, and 5% TS. Samples of this second hydrolysate were stored at −20 °C until required.

**Quantification of Degree of Hydrolysis.** The degree of hydrolysis (DH%), defined as the percentage of peptide bonds cleaved, was calculated from the volume and molarity of NaOH used to maintain the pH constant using the formula[14] (eq 1)

$$\mathrm{DH\%} = 100 \times B \times N_b \times \left(\frac{1}{\alpha}\right) \times \left(\frac{1}{\mathrm{MP}}\right) \times \left(\frac{1}{h_{\mathrm{total}}}\right) \quad (1)$$

where $B$ is the base consumption in mL, $N_b$ the normality of the base, $\alpha$ the average degree of dissociation of the $\alpha$-$NH_2$ groups, MP the mass of protein being hydrolyzed (g), and $h_{\mathrm{total}}$ the total number of peptide bonds in the protein substrate (8.8 mequiv/g protein).

**Determination of Enzyme Activity.** The general proteinase activity in hydrolysates was determined by assay with azocasein.[23] The assay was performed by the addition of 100 $\mu$L of the hydrolysate solution to 1 mL of 0.5% (w/v) azocasein dissolved in 50 mM phosphate buffer, pH 7.0. After 10 min incubation at 50 °C, the reaction was terminated by the addition of 400 $\mu$L of 24% (w/v) trichloroacetic acid followed by centrifugation at 14000 rpm for 5 min (Hettich, Universal 320R, Tuttlingen, Germany). A sample of supernatant (500 $\mu$L) was removed and added to a cuvette containing 500 $\mu$L of 1 M NaOH. Absorbance at 440 nm was measured using an UV mini-1240 UV−vis spectrophotometer Shimadzu (Duisburg, Germany). Activity was expressed as the change in absorbance (ΔAbs) at 440 nm per minute per milligram of enzyme.

**Heat Inactivation of Corolase PP in WPC Hydrolysates.** The heat inactivation kinetics were determined in 4.0 mL aliquots at different TS concentrations (30, 20, 10, and 5%) when Corolase PP was readded to the first WPC hydrolysate. The proteolytic preparation was added at room temperature at (i) a constant concentration of 1 mg/mL or (ii) at an enzyme/substrate of 0.3% (w/w) to each solution. The rate of thermal inactivation was then studied on immediate incubation at constant temperature of 65, 67, 70, or 75 °C at atmospheric pressure using a temperature-controlled (±0.1 °C) water bath (Lauda, Lauda-Königshofen, Germany). Aliquots (0.5 mL) of the heated sample were removed at different intervals for each incubation temperature and immediately cooled by immersion in an ice−water bath. The residual enzyme activity in the samples was measured using the azocasein assay.

The kinetics of Corolase PP thermal inactivation was also assessed in the second hydrolysate. In this case the enzyme was added to 4 mL aliquots of the 30, 20, 10, and 5% TS second hydrolysate at an E:S of 0.3% (w/w), as described above. The thermal inactivation kinetics were then studied at 63, 65, 67, and 70 °C for the 30 and 20% TS solutions and at 60, 63, 65, and 67 °C for the 10 and 5% TS solutions, as described above.

**Heat Inactivation Kinetic Analysis.** The inactivation process for an enzyme can be described by the general equation (eq 2)

$$-\frac{\mathrm{d}A}{\mathrm{d}t} = kA^n \quad (2)$$

where $-\mathrm{d}A/\mathrm{d}t$ represents the rate of enzyme inactivation, $k$ the rate constant, $A$ the activity at each time, and $n$ the reaction order.

For a first-order reaction, $n = 1$ (eq 3)

$$-\frac{\mathrm{d}A}{\mathrm{d}t} = kA; \; -\frac{\mathrm{d}A}{A} = k\,\mathrm{d}t \quad (3)$$

and integrating (eq 4)

$$\ln\left(\frac{A_t}{A_0}\right) = -kt \quad (4)$$

where $A_0$ is the initial activity, $A_t$ is the remaining activity at time $t$, and $k$ is the inactivation rate constant at a given temperature. When the inactivation process follows first-order kinetics, the graphical representation of eq 4 gives a straight line and the value $b$ of the ordinate intercept (time $t = 0$) is close to zero. The inactivation rate constant $k$ can be estimated by linear regression analysis of the natural logarithm of residual activity versus treatment time.

The $D$ values (time required for 90% reduction of the initial activity) were calculated in relation to the $k$ values by eq 5:

$$D = \frac{1}{k} \quad (5)$$

The effect of temperature on $D$ value was also studied, and the $z$ value (temperature necessary to reduce $D$ value by 1 logarithmic unit) was calculated by regression analysis of the line obtained on plotting the logarithm of the $D$ value against the corresponding temperatures. The $z$ value corresponds to the reciprocal of the slope of this line.

Arrehenius's law is usually utilized to describe the temperature dependence of $k$ values, and it is algebraically expressed as per eq 6

$$\ln k = \ln k_0 - \frac{E_a}{RT} \quad (6)$$

where $k_0$ is the Arrhenius constant, $E_a$ the activation energy (kJ/mol), $R$ the universal gas constant (8.314 J/mol·K), and $T$ the absolute temperature (K). When the natural logarithm of the rate constant versus the reciprocal of the absolute temperature is plotted according to eq 5, the activation energy ($E_a$) value can be obtained from the slope.

The values for $E_a$ and inactivation rate constant ($k$) allowed for the determination of different thermodynamic parameters such as change in enthalpy ($\Delta H$), entropy ($\Delta S$), and free energy of activation ($\Delta G$), respectively, using the following equations (eqs 7, 8, and 9):

$$\Delta G = -RT \ln\left(\frac{kh_p}{K_B T}\right) \quad (7)$$

$$\Delta H = E_a - RT \quad (8)$$

$$\Delta S = \left(\frac{\Delta H - \Delta G}{T}\right) \quad (9)$$

$K_B$ is the Boltzmann constant ($1.3806 \times 10^{-23}$ J/K), $h_p$ is Planck's constant ($6.6262 \times 10^{-34}$ J·s), and $k$ is the inactivation rate constant at each temperature ($s^{-1}$).

**Gel Permeation HPLC of Whey Protein Hydrolysates.** Gel permeation high-performance liquid chromatography (GP-HPLC) was

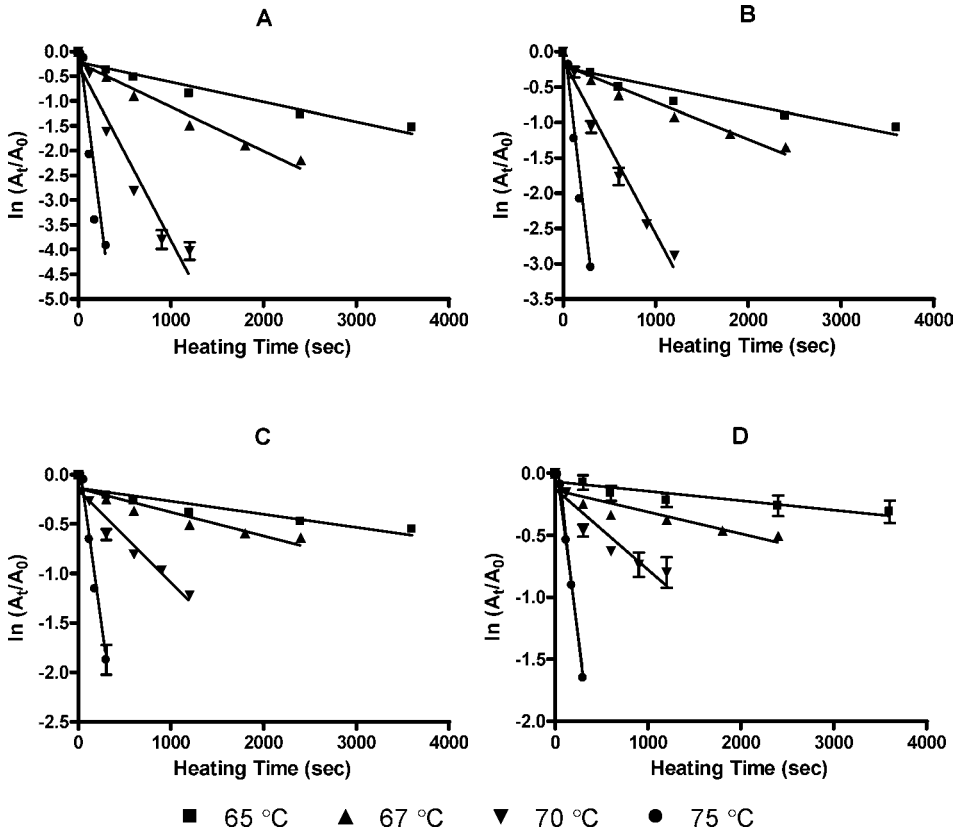


**Figure 1.** Thermal inactivation curves for Corolase PP at 1 mg/mL in the temperature range of 65−75 °C at a total solids (TS) concentration of (A) 5, (B) 10, (C) 20, and (D) 30% in a whey protein hydrolysate having a degree of hydrolysis (DH) of ∼10%. The residual activity at each time point was determined by the azocasein assay. $A_0$ and $A_t$ are initial and residual activities at the time measured, respectively. Each data point is the mean of six determinations from two independent experiments. Vertical bars represent standard deviations.

performed on the whey protein hydrolysate and intact whey protein samples using a Waters HPLC system (Waters, Milford, MA, USA). This was composed of a model 1525 binary pump, a model 717 Plus autosampler, and a model 2487 dual wavelength absorbance detector interfaced with a Breeze data-handling package. The mobile phase was 30% (v/v) acetonitrile containing 0.1% (v/v) TFA. Samples were diluted in mobile phase to 0.25% (w/v) protein, filtered through 0.2 $\mu$m syringe filters. A volume of 20 $\mu$L was applied to a TSK G2000 SW separating column (600 × 7.5 mm i.d.) connected to a TSKGEL SW guard column (75 × 7.5 mm i.d.). Isocratic elution with the mobile phase at a flow rate of 0.5 mL/min was used for separation, and it was monitored at 214 nm. A calibration curve was prepared from the average retention times of standard proteins and peptides.[24] The void volume ($V_0$) was estimated with thyroglobulin (600,000 Da) and the total column volume ($V_t$) was estimated with L-tyrosine HCl (218 Da).

**Determination of Apparent Viscosity.** Apparent viscosity ($\eta_{app}$) was measured using a Brookfield programmable DV-II+ viscosimeter (Brookfield Engineering Laboratories, Middleboro, MA, USA) fitted with an ultralow (UL) adaptor. The UL adaptor was connected to a Lauda circulating water bath (Lauda-Königshofen, Germany) by a ULA-40Y water jacket. $\eta_{app}$ values were measured at 25 °C and pH 7.0 at various shear rates.

**Statistical Analysis.** Mean and standard deviations were calculated for all data. Data were statistically evaluated by analysis of variance (ANOVA) according to the Bonferroni post-test or $t$ test using GraphPad Prism 4 software.

## RESULTS AND DISCUSSION

Enzymatic hydrolysis of food proteins is the most widely used approach for the generation of hydroysates having enhanced techno- and biofunctional properties.[1,2,25] Hydrolysis is carried out under defined conditions of pH, temperature, time, and E:S ratio, and at the end of the process the enzyme used is inactivated, commonly using a thermal treatment step. However, various chemical (such as Maillard reaction product formation) and physical (protein/peptide denaturation) changes have been described following thermal processing. As a consequence, heat treatment of the hydrolysate may affect the functional, nutritional, and biological properties of the peptides therein.[21,22] Therefore, optimization of the heat treatment step with regard to the techno- and biofunctional properties of the peptides while minimizing the overall energy input associated with the thermal treatment step is of significant interest to the food industry.

The effect of the TS concentration on the heat inactivation kinetics of Corolase PP during the generation of WPC hydrolysates was therefore investigated. A 30% (w/v) aqueous solution of WPC was hydrolyzed with Corolase PP using the conditions already outlined under Materials and Methods. After 4 h of hydrolysis, the DH reached was 9.7% as determined by the amount of NaOH consumed to maintain constant pH.[14] The hydrolysate solution was subjected to a heat treatment of 80 °C for 20 min to ensure complete inactivation of the enzyme, as confirmed by the azocasein assay. The hydrolysate at 30% TS was then diluted in distilled water to yield solutions containing 5, 10, and 20% TS. Corolase PP was added to each solution at (i) a concentration of 1 mg/mL or at (ii) an E:S of 0.3% (w/w in terms of protein) for a detailed kinetic study of thermal inactivation over a range of temperatures from 65 to 75 °C with different holding times.

Figures 1 and 2 present the thermal inactivation curves for Corolase PP as semilog plots at each TS concentration for the

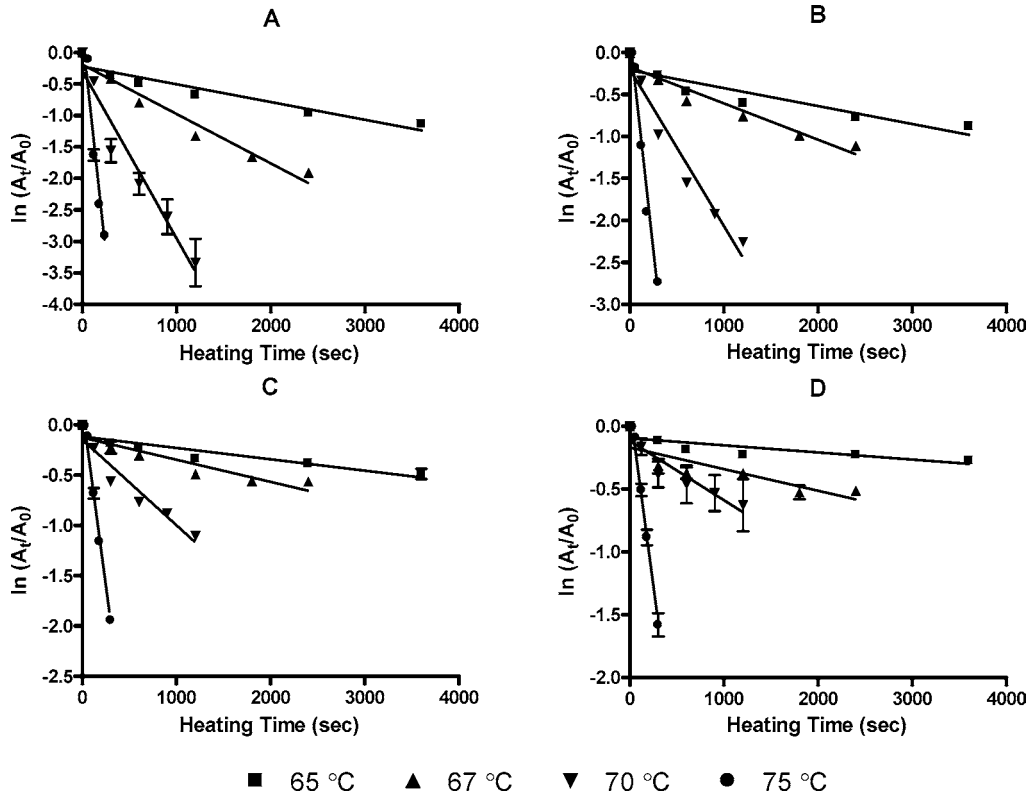


**Figure 2.** Thermal inactivation curves for Corolase PP at an enzyme to substrate ratio of 0.3% (w/w) in the temperature range of 65−75 °C at a total solids (TS) concentration of (A) 5, (B) 10, (C) 20, and (D) 30% in a whey protein concentrate hydrolysate having a degree of hydrolysis (DH) of ∼10%. The residual activity at each time point was determined by the azocasein assay. $A_0$ and $A_t$ are initial and residual activities at the time measured, respectively. Each data point is the mean of six determinations from two independent experiments. Vertical bars represent standard deviations.

**Table 1. Time Required for 90% Inactivation (*D* Value) and Temperature Required To Reduce the *D* Value by 1 Log (*z* Value) during the Thermal Inactivation of Corolase PP in a Whey Protein Concentrate Hydrolysate having a Degree of Hydrolysis of ∼10%[a]**

| | | *D* value (h) | | | |
|---|---|---|---|---|---|
| | TS (%) | 65 °C | 67 °C | 70 °C | 75 °C |
| Corolase PP at 1 mg/mL | 5 ($z$ = 6.4 °C) | 20.8 a/e/h | 9.3 b/e/h | 2.4 c/e/h | 0.6 d/e/h |
| | 10 ($z$ = 6.2 °C) | 27.8 a/e/h | 16.7 b/ef/h | 3.7 c/e/h | 0.7 d/e/h |
| | 20 ($z$ = 5.7 °C) | 83.3 a/f/h | 33.3 b/f/h | 10.4 c/e/h | 1.4 d/e/h |
| | 30 ($z$ = 5.3 °C) | 111.1 a/g/h | 41.7 b/g/h | 13.9 c/e/h | 1.4 d/e/h |
| Corolase PP:substrate ratio 0.3% (w/w) | 5 ($z$ = 6.0 °C) | 27.8 a/e/i | 10.4 b/e/i | 2.5 c/e/h | 0.6 d/e/h |
| | 10 ($z$ = 5.8 °C) | 41.7 a/e/i | 18.5 b/ef/h | 4.5 c/e/h | 0.8 d/e/h |
| | 20 ($z$ = 5.3 °C) | 83.3 a/f/h | 41.7 b/f/h | 9.8 c/e/h | 1.2 d/e/h |
| | 30 ($z$ = 5.2 °C) | 138.9 a/g/h | 41.7 b/g/h | 18.5 c/g/h | 1.5 d/g/h |

[a]Mean of six replicates from two independent experiments. Similar letters (a−d) within a row indicate no significant difference ($P > 0.05$). Similar letters within a column for each concentration of Corolase PP (e−g) indicate no significant difference ($P > 0.05$), and similar letters within a column for the same TS concentration between different Corolase PP concentrations (h, i) indicate no significant difference ($P > 0.05$).

enzyme when added at 1 mg/mL or at an E:S of 0.3% (w/w), respectively. As expected, the rate of enzyme inactivation increased with temperature and treatment time. This has previously been described for the heat inactivation of other enzymes such as lactoperoxidase[26] and polyphenol oxidase.[18] Figures 1 and 2 also show that the rate of inactivation was influenced by the TS content in solution, being higher at lower TS concentrations. No previous studies, in the peer-reviewed

literature, appear to have reported on the thermal inactivation of proteolytic preparations during the generation of protein hydrolysates at different TS values. Noticeably, despite the fact that Corolase PP is a mixture of different enzyme activities (trypsin, chymotrypsin, elastase, and exopeptidases),[27] the semilog plots were linear at all temperatures studied (65, 67, 70, and 75 °C). The enzyme inactivation plots at different TS values had coefficients of correlation >0.8, and the value for *b* (the ordinate intercept) was close to zero. This is consistent with thermal inactivation by means of a first-order reaction.

The inactivation rate constants (*k*) were calculated (data not shown) from the slopes of these lines. The *k* values increased with heating temperature, indicating that Corolase PP is less thermostable at higher temperatures. This trend was observed when Corolase PP was added at a constant concentration (1 mg/mL) and at a constant E:S (0.3% (w/w)).

For further comparison of the results, the *D* and *z* values were calculated as described under Materials and Methods, and the results are shown in Table 1. Increasing the temperature from 65 to 75 °C resulted in a significant decrease in the *D* value at all TS concentrations ($P < 0.001$). By comparison of the same temperature at different TS concentrations, it was observed that the *D* value was generally significantly higher at higher TS concentration. For example, the *D* values at 65 °C for 5, 10, 20, and 30% TS when the enzyme was added at 1 mg/mL were 20.8, 27.8, 83.3, and 111.1 h, respectively (Table 1). This indicates that the enzyme was more thermostable in higher TS concentrations. Moreover, in general, the mean *D* value was also higher when the enzyme was added at an E:S of 0.3% (w/w) than when it was added at the same concentration of 1 mg/mL. It was significantly different on heating at 65 °C for the 5 and 10% TS ($P < 0.001$) and at 67 °C at 5% TS samples ($P < 0.05$). The *z* value was lower at higher TS, and by comparison of the same TS level, it was higher when the enzyme was added at 1 mg/mL. Differences have previously been reported in the thermoresistance of lactoperoxidase, being more stable in milk than in diluted whey.[26] Furthermore, alkaline phosphatase was reported to be more heat resistant in milk than in buffer.[28] These findings are in agreement with the results herein, where it appears that higher TS solutions have a protective effect on the enzyme leading to higher heat treatments being necessary for its inactivation.

The dependence of the inactivation rate constant on temperature was assessed using the Arrhenius equation giving correlation coefficients >0.96 (Figure 3). This linearity is an indication that inactivation occurs through a unique mechanism dependent on temperature, such as protein unfolding.[29] The slope of the Arrhenius plots allowed estimation of the activation energy ($E_a$) value for each TS concentration at the two different levels of Corolase PP addition (Table 2). In agreement with the trend for the *D* values, increasing the TS concentration resulted in an increase in $E_a$.

The thermodynamic parameters of inactivation were also calculated, and these are shown in Table 2. These parameters include $\Delta G$, the Gibbs free energy change (the energy barrier for enzyme inactivation); $\Delta H$, the enthalpy change (indicative of the number of bonds broken during inactivation); and $\Delta S$, the entropy change (indicative of enzyme and solvent disorder). The value for $\Delta G$ was similar for all temperatures studied, the different TS, and the enzyme addition level. Similar to the trend observed in the $E_a$ and *D* values, the $\Delta H$ and $\Delta S$ values increased with increasing TS and were higher when the enzyme was added at an E:S of 0.3% (w/w) than when added at 1 mg/mL. The amount of enzyme in the 5, 10, 20, and 30% TS solutions when

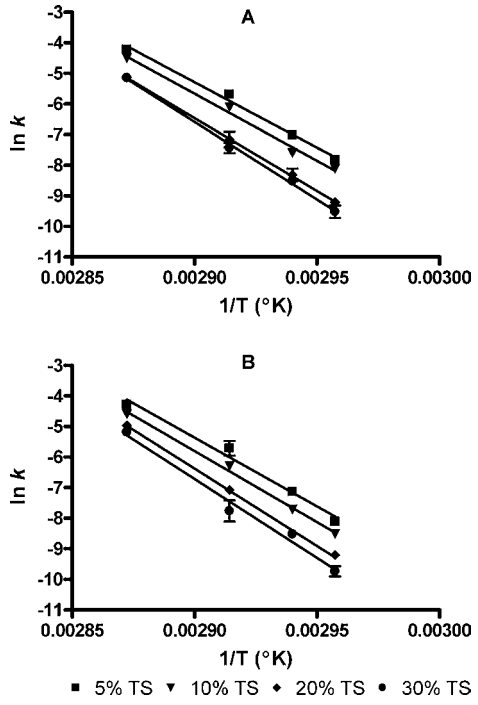


**Figure 3.** Arrhenius plots showing the effect of temperature on the rate constant for the thermal inactivation of Corolase PP at (A) 1 mg/mL and (B) an enzyme to substrate ratio of 0.3% (w/w) at different total solids (TS) concentrations in the whey protein concentrate hydrolysate with a DH of ~10%. Each point is the mean of six determinations from two different experiments. Vertical bars represent standard deviations.

added at an E:S of 0.3% (w/w) was estimated to be equivalent to 0.22, 0.45, 0.90, and 1.37 mg/mL, respectively. The above findings may indicate that the composition of the solution, and not only the TS alone, has a role in the thermal inactivation of the enzyme. To further investigate this, the 30% TS hydrolysate generated at an E:S of 0.3 (w/w) was subjected to further hydrolysis with Corolase PP using the conditions outlined under Materials and Methods. The DH increased by 11.44% after 6 h of incubation, giving a final DH in the sample of 21.2% when the extent of hydrolysis achieved during the first hydrolysis step was considered. The hydrolysate solution was subjected to heat treatment of 80 °C for 20 min to ensure complete inactivation of the enzyme, as confirmed by the azocasein assay. Gel permeation chromatography was also performed to confirm the higher extent of hydrolysis following the second hydrolysis step. The molecular mass distribution profiles shown in Figure 4 demonstrate that the amount of intact protein decreased with the increased extent of hydrolysis. Furthermore, as expected, the second hydrolysate had a higher amount of small peptides (<1 kDa) in comparison to the first hydrolysate.

The 21.2% DH 30% TS hydrolysate was diluted to 20, 10, and 5% TS, and the heat inactivation kinetics of Corolase PP were subsequently studied following enzyme addition at an E:S of 0.3% (w/w in terms of protein). Temperature ranges from 60 to 67 °C for the 5 and 10% TS and from 63 to 70 °C for the 20 and 30% TS with different heating times were used for these studies. Interestingly, it was not possible to use the same range of temperatures as those used in studying the thermal inactivation of the first hydrolysate (DH ~10%) as the enzyme was found to be more heat sensitive in the higher DH (~21%) hydrolysate, particularly at lower TS. The thermal inactivation curves obtained are shown in Figure 5. As for the lower DH hydrolysate,

**Table 2. Thermodynamic Parameters for Heat Inactivation of Corolase PP in Whey Protein Concentrate Hydrolysates with a Degree of Hydrolysis Value of ~10% at Different Total Solids (TS)[a]**

| | | Corolase PP (1 mg/mL) | | | Corolase PP (E:S 0.3% (w/w)) | | |
|---|---|---|---|---|---|---|---|
| TS (%) | temp (°C) | $\Delta H$ (kJ/mol) | $\Delta G$ (kJ/mol) | $\Delta S$ (J/mol·K) | $\Delta H$ (kJ/mol) | $\Delta G$ (kJ/mol) | $\Delta S$ (J/mol·K) |
| 5 | | | $E_a$ = 354 ± 4 kJ/mol a/e | | | $E_a$ = 374 ± 3 kJ/mol a/f | |
| | 65 | 352 ± 3 | 105 ± 0 | 729 ± 9 | 371 ± 2 | 106 ± 0 | 784 ± 6 |
| | 67 | 352 ± 3 | 103 ± 0 | 730 ± 9 | 371 ± 2 | 104 ± 0 | 785 ± 6 |
| | 70 | 352 ± 3 | 101 ± 0 | 731 ± 9 | 371 ± 2 | 101 ± 1 | 787 ± 8 |
| | 75 | 352 ± 3 | 97 ± 0 | 729 ± 9 | 371 ± 2 | 98 ± 0 | 783 ± 6 |
| 10 | | | $E_a$ = 365 ± 3 kJ/mol a/e | | | $E_a$ = 387 ± 4 kJ/mol b/f | |
| | 65 | 362 ± 2 | 106 ± 0 | 757 ± 7 | 384 ± 3 | 107 ± 0 | 819 ± 8 |
| | 67 | 362 ± 2 | 105 ± 0 | 754 ± 7 | 384 ± 3 | 105 ± 0 | 819 ± 7 |
| | 70 | 362 ± 2 | 102 ± 0 | 757 ± 8 | 384 ± 3 | 102 ± 0 | 821 ± 8 |
| | 75 | 362 ± 2 | 99 ± 0 | 755 ± 7 | 384 ± 3 | 99 ± 0 | 819 ± 8 |
| 20 | | | $E_a$ = 396 ± 3 kJ/mol b/e | | | $E_a$ = 422 ± 2 kJ/mol c/f | |
| | 65 | 393 ± 2 | 109 ± 0 | 841 ± 7 | 420 ± 1 | 109 ± 0 | 919 ± 2 |
| | 67 | 393 ± 2 | 107 ± 1 | 841 ± 9 | 420 ± 0 | 108 ± 0 | 917 ± 2 |
| | 70 | 393 ± 2 | 105 ± 1 | 841 ± 9 | 420 ± 0 | 105 ± 0 | 919 ± 2 |
| | 75 | 393 ± 2 | 101 ± 0 | 841 ± 7 | 420 ± 0 | 100 ± 0 | 918 ± 1 |
| 30 | | | $E_a$ = 422 ± 19 kJ/mol c/e | | | $E_a$ = 429 ± 3 kJ/mol d/e | |
| | 65 | 420 ± 13 | 110 ± 1 | 916 ± 38 | 428 ± 1 | 110 ± 0 | 938 ± 3 |
| | 67 | 420 ± 13 | 108 ± 0 | 917 ± 40 | 428 ± 1 | 108 ± 0 | 940 ± 4 |
| | 70 | 420 ± 13 | 106 ± 0 | 915 ± 38 | 428 ± 1 | 107 ± 1 | 936 ± 1 |
| | 75 | 420 ± 13 | 101 ± 0 | 916 ± 39 | 428 ± 1 | 101 ± 0 | 939 ± 3 |

[a]Mean of six replicates ± SD from two independent experiments. For $E_a$, similar letters (a–d) within a column indicate no significant difference ($P > 0.05$), similar letters (e, f) within a row indicate no significant difference ($P > 0.05$).

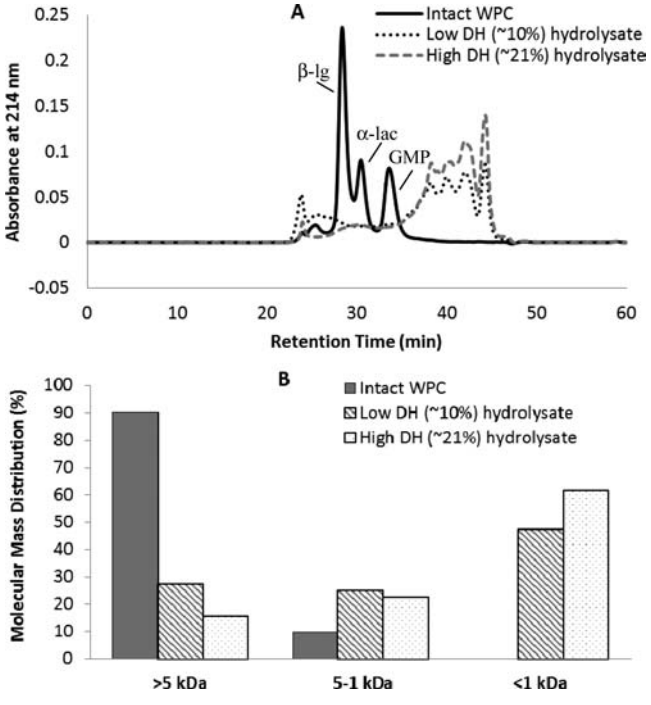


**Figure 4.** Gel permeation high-performance liquid chromatography (GP-HPLC) profiles (A) and molecular mass distributions (B) of intact whey protein concentrate (WPC) and WPC hydrolysate at degree of hydrolysis (DH) of ~10 and ~21%. Abbreviations: $\beta$-lg, $\beta$-lactoglobulin; $\alpha$-lac $\alpha$-lactalbumin; GMP, glycomacropeptide.

enzyme inactivation increased with increasing temperature and treatment time. Moreover, this inactivation rate was influenced by the TS content in solution, being higher at lower TS. The inactivation rate constants ($k$) were also calculated from the slopes of these lines (data not shown). The $k$ values increased with heating temperature, again indicating that Corolase PP was less thermostable at higher temperatures.

The $D$ and $z$ values were calculated for Coralase PP thermal inactivation in the higher DH hydrolysate sample, and the results are shown in Table 3. Increasing the temperature resulted in a significant ($P < 0.001$) decrease in $D$ value at all TS concentrations. When the same thermal inactivation temperature was compared at different TS concentration, it was observed that the $D$ value was significantly higher at higher TS concentration, indicating that the enzyme is more thermostable at higher TS. Moreover, comparing the $D$ values at the same temperature (65, 67, and 70 °C) with those obtained for the lower DH (~10%) hydrolysate shows that $D$ values were significantly lower for the higher DH (~21%) hydrolysate ($P < 0.05$). For example, the $D$ values for the enzyme at an E:S 0.3% (w/w) at 65 °C at 30% TS were 138.9 and 41.7 h in the low DH (~10%) and high DH (~21%) hydrolysate, respectively (Tables 1 and 3). This indicates that Corolase PP was more heat sensitive in the higher DH hydrolysate, which contains a higher level of low molecular mass peptides, at the same TS compared to the lower DH hydrolysate. Apart from the 5% TS sample, the $z$ values were lower in the higher TS solutions. In addition, these values were lower in the high DH as opposed to the low DH samples (Table 3).

When the natural logarithm of the rate constant ($k$) was plotted against the reciprocal of the absolute temperature (K), a linear relationship, with correlation coefficients >0.98, was observed in the temperature range studied (Figure 6). The $E_a$ value for each TS concentration was calculated from the slope of

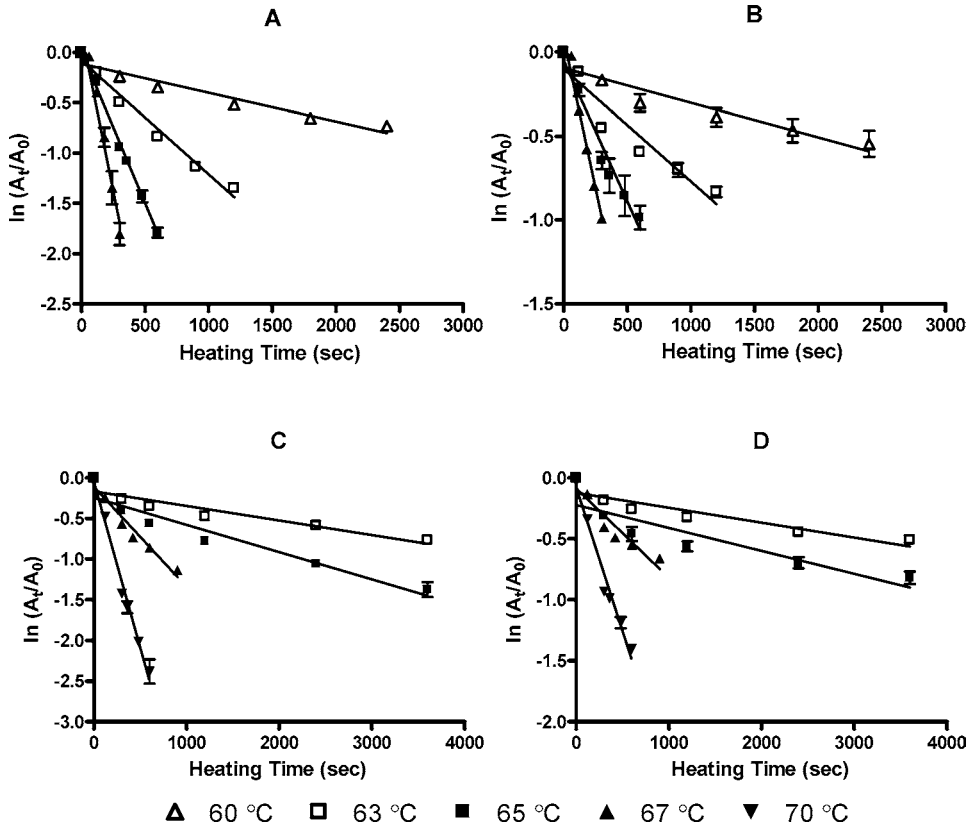


**Figure 5.** Thermal inactivation curves for Corolase PP at an enzyme to substrate ratio of 0.3% (w/w) in the temperature range of 60−70 °C at a total solids (TS) concentration of (A) 5, (B) 10, (C) 20, and (D) 30% in a whey protein concentrate hydrolysate having a degree of hydrolysis (DH) of ∼21%. The residual activity at each time point was determined by the azocasein assay. $A_0$ and $A_t$ are initial and residual activities at the time measured, respectively. Each data point is the mean of six determinations from two independent experiments. Vertical bars represent standard deviations.

**Table 3. Time Required for 90% Inactivation (*D* Value) and Temperature Required To Reduce the *D* Value by 1 Log (*z* Value) during the Thermal Inactivation of Corolase PP in Whey Protein Concentrate Hydrolysates with a Degree of Hydrolysis of ∼21%[a]**

| | | *D* values (h) | | | | |
|---|---|---|---|---|---|---|
| | TS (%) | 60 °C | 63 °C | 65 °C | 67 °C | 70 °C |
| Corolase PP:substrate ratio 0.3% (w/w) | 5 ($z$ = 5.1 °C) | 27.8 a/e | 7.2 b/e | 2.8 c/e | 1.2 d/e | nd[b] |
| | 10 ($z$ = 5.5 °C) | 41.7 a/f | 12.8 b/f | 5.2 c/ef | 2.3 d/ef | nd |
| | 20 ($z$ = 5.2 °C) | nd | 41.7a/g | 23.8 b/f | 6.9c/fg | 2.1 d/e |
| | 30 ($z$ = 4.9 °C) | nd | 83.3 a/h | 41.7 b/g | 11.9 c/g | 3.5d/e |

[a]Mean of six replicates from two independent experiments. Similar letters (a−d) within a row indicate no significant difference ($P > 0.05$). Similar letters within a column (e−g) indicate no significant difference ($P > 0.05$). [b]Not determined.

these plots (Table 4). Again, apart from the 5% TS sample, the $E_a$ value increased with increasing TS. Table 4 also shows the thermodynamic parameters for the inactivation of Corolase PP in the high DH hydrolysate. As was also observed in the case of the low DH hydrolysate, the value for $\Delta G$ was similar for all temperatures and the different TS. As was the case with the $E_a$ values, $\Delta H$ and $\Delta S$ increased with increasing TS, except in the case of the 5% TS sample.

The results obtained for the higher DH hydrolysate indicate that the enzyme was more heat resistant at the higher TS concentration. These results are consistent with those found for the lower DH hydrolysate. As already outlined, differences in enzyme thermostability depending on the media composition have been reported in the literature. Additional examples include glutamyl transferase, which was reported to be more stable in skimmed milk than in whole milk or cream.[30] Furthermore, lactoperoxidase was shown to display different thermostabilities in goat's, sheep's, and cow's milk.[20] An interesting finding herein has been that the heat resistance of the enzyme (Corolase PP) was significantly lower ($P < 0.05$) in the higher DH (∼21%) hydrolysate when compared with that in the low DH (∼10%) hydrolysate at similar TS levels. This demonstrates that not only does the TS present in the solution influence enzyme inactivation, but it is also influenced by the type of components present. A possible explanation for these observations may be related to differences in the viscosity between the hydrolysates. It


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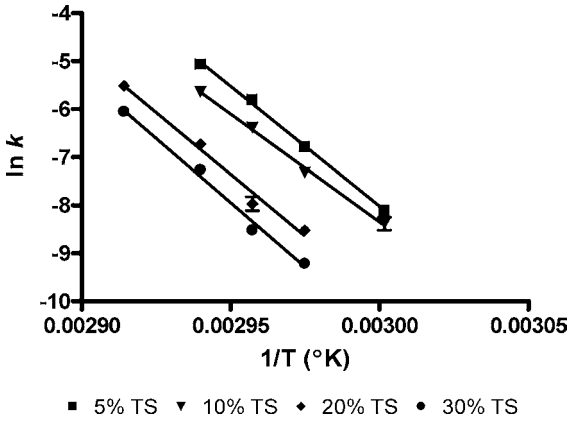


**Figure 6.** Arrhenius plots showing the effect of temperature on the rate constant for the thermal inactivation of Corolase PP at an enzyme to substrate ratio of 0.3% (w/w), at different total solids (TS) concentrations in a whey protein concentrate hydrolysate having a degree of hydrolysis (DH) of ∼21%. Each point is the mean of six determinations from two different experiments. Vertical bars represent standard deviations.

**Table 4. Thermodynamic Parameters for Heat Inactivation of Corolase PP Whey Protein Concentrate Hydrolysates with a Degree of Hydrolysis Value of ∼21% at Different Total Solids (TS)[a]**

| | | Corolase PP (E:S 0.3% (w/w)) | | |
|---|---|---|---|---|
| TS (%) | temp (°C) | $\Delta H$ (kJ/mol) | $\Delta G$ (kJ/mol) | $\Delta S$ (J/mol·K) |
| 5 | | $E_a$ = 414 ± 17 kJ/mol a | | |
| | 60 | 421 ± 4 | 104 ± 0 | 950 ± 14 |
| | 63 | 421 ± 4 | 102 ± 0 | 950 ± 14 |
| | 65 | 421 ± 4 | 99 ± 0 | 950 ± 14 |
| | 67 | 421 ± 4 | 98 ± 0 | 950 ± 14 |
| 10 | | $E_a$ = 374 ± 33 kJ/mol b | | |
| | 60 | 390 ± 5 | 105 ± 0 | 853 ± 16 |
| | 63 | 390 ± 5 | 103 ± 0 | 852 ± 17 |
| | 65 | 390 ± 5 | 101 ± 0 | 853 ± 17 |
| | 67 | 390 ± 5 | 100 ± 0 | 853 ± 16 |
| 20 | | $E_a$ = 428 ± 3 kJ/mol ac | | |
| | 63 | 425 ± 2 | 107 ± 0 | 948 ± 6 |
| | 65 | 425 ± 2 | 106 ± 0 | 945 ± 7 |
| | 67 | 425 ± 2 | 103 ± 0 | 947 ± 6 |
| | 70 | 425 ± 2 | 100 ± 0 | 946 ± 6 |
| 30 | | $E_a$ = 446 ± 4 kJ/mol c | | |
| | 63 | 443 ± 3 | 108 ± 0 | 995 ± 8 |
| | 65 | 443 ± 3 | 107 ± 0 | 993 ± 8 |
| | 67 | 443 ± 3 | 104 ± 0 | 994 ± 8 |
| | 70 | 443 ± 3 | 102 ± 0 | 994 ± 8 |

[a]Mean of six replicates ± SD from two independent experiments. For the $E_a$, similar letters indicate no significant difference ($P > 0.05$).

is known that viscosity can be affected by the amount of TS, as previously described for soy protein, micellar casein,[31] and WPC.[15] In addition, it is also affected by the type of species present in solution.[31] The apparent viscosity of the low and high DH hydrolysates was measured at 25 °C (Figure 7) using shear rates from 6.12 to 97.8 $s^{-1}$ (it was not possible to compare the values at equivalent shear rates due to the large differences in viscosity). The low DH hydrolysate at 30% TS was too viscous to

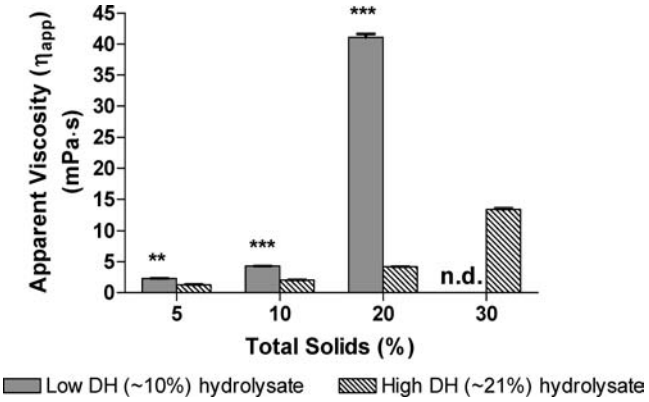


**Figure 7.** Apparent viscosity ($\eta_{app}$) of the hydrolysates with a degree of hydrolysis (DH) of ∼10% and with a DH of ∼21% at different total solids (TS) concentrations. "n.d." means not determined. Significant difference between the low and high DH hydrolysate for ** ($P < 0.01$) or *** ($P < 0.001$).

measure the viscosity. As expected, it was found that the viscosity increased with the amount of TS in solution for both hydrolysates. Moreover, a significantly lower viscosity was observed in the high DH (∼21%) compared with the low DH (∼10%) hydrolysate at all equivalent TS concentrations ($P < 0.01$). This decrease in viscosity in the high DH hydrolysate could be due to the lower amount of intact protein present in solution as shown by gel permeation chromatography. The major whey proteins, $\beta$-lactoglobulin, $\alpha$-lactalbumin, immunoglobulins, and bovine serum albumin, denature around 78, 62, 72, and 64 °C, respectively.[32] Whereas some aggregation of residual intact proteins may have occurred after the heat treatment of 80 °C for 20 min, it is likely that some enzyme-induced peptide aggregation took place. This is supported by the observation that new peaks of high molecular mass (eluting between 22 and 27 min) were observed in the hydrolysates after heat treatment. This aggregation may contribute to solution viscosity.[33] Moreover, it has been observed that the heat transfer coefficient in a solution decreases with increasing viscosity.[34,35] This may explain the fact that in the high DH hydrolysate the enzyme is more heat sensitive as the viscosity is significantly lower than in the low DH hydrolysate. In addition, this may also explain the higher heat resistance of the enzyme at higher TS due to the associated higher viscosity.

On the other hand, not all of the differences in the heat sensitivity of Corolase PP can be attributed to differences in solution viscosity. When the $D$ values at the same temperature and TS were compared and also when the $E_a$ values obtained at similar TS were compared, it was found that there were significant differences in these parameters depending on whether the enzyme was added at 1 mg/mL or at an E:S of 0.3% (w/w). However, the viscosity values were similar for these solutions. The concentrations of enzyme in the 5 and 10% TS suspensions when Corolase PP was added at an E:S of 0.3% (w/w) were 0.22 and 0.45 mg/mL, respectively. These values were lower when enzyme was included at a constant concentration (1 mg/mL). However, the $D$ values obtained during inactivation at 65 °C for the 5 and 10% TS and at 67 °C for the 5% TS, as well as the $E_a$ values at 5 and 10% TS, were significantly higher ($P < 0.05$) in the solutions containing the lower concentration of enzyme. This could be explained by the fact that although the TS and unhydrolyzed protein levels were the same, the level of enzyme in solution was different. When there was less enzyme in solution, it

could be postulated that probably all, or at least a major portion, was bound to the cleavage sites on the intact protein, and this could have a thermoprotective effect. On the other hand, when more enzyme was in the solution, more "free" enzyme could be available that was not bound to the protein substrate, and therefore in this situation it could be more susceptible to heat inactivation.

In summary, the data obtained herein indicate that the best option to reduce the level of thermal treatment applied to the WPC hydrolysate sample to ensure efficient enzyme inactivation would be to employ low solution TS values combined with a high sample DH. Using those parameters, the results show that the viscosity of the solution is lower and the enzyme is more heat sensitive. Up to now, no detailed information appears to exist in the scientific literature in respect to the thermal inactivation behavior of proteolytic preparations used in the food industry. Therefore, the findings herein may be useful in the industrial context because heat treatments may adversely affect different characteristics of the final product, such as its nutritional quality[36] or biological activity.[22,37] Furthermore, the results demonstrate that adoption of appropriate hydrolysis conditions may prove more cost efficient from both functionality and processing perspectives. The results herein apply to the thermal inactivation of Corolase PP in a whey protein hydrolysate; the thermal inactivation kinetics of other enzyme−substrate combinations would need to be determined on a case-by-case basis.

## AUTHOR INFORMATION


### Corresponding Author

*(R.J.F.) Phone: +353 61 202598. Fax: +353 61 331490. E-mail: dick.fitzgerald@ul.ie.



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## REFERENCES


(1) Foegeding, E. A.; Davis, J. P.; Doucet, D.; McGuffey, M. K. Advances in modifying and understanding whey protein functionality. *Trends Food Sci. Technol.* **2002**, *13*, 151−159.

(2) Clemente, A. Enzymatic protein hydrolysates in human nutrition. *Trends Food Sci. Technol.* **2000**, *11*, 254−262.

(3) Korhonen, H. Milk-derived bioactive peptides: from science to applications. *J. Funct. Foods* **2009**, *1*, 177−187.

(4) Agyei, D.; Danquah, M. K. Industrial-scale manufacturing of pharmaceutical-grade bioactive peptides. *Biotechnol. Adv.* **2011**, *29*, 272−277.

(5) Panyam, D.; Kilara, A. Enhancing the functionality of food proteins by enzymatic modification. *Trends Food Sci. Technol.* **1996**, *7*, 120−125.

(6) Slattery, H.; FitzGerald, R. J. Functional properties and bitterness of sodium caseinate hydrolysates prepared with a *Bacillus* proteinase. *J. Food Sci.* **1998**, *63*, 418−422.

(7) Bouchier, P. J; FitzGerald, R. J.; O'Cuinn, G. Hydrolysis of $\alpha_{s1}$- and $\beta$-casein-derived peptides with a broad specificity aminopeptidase and proline specific aminopeptidases from *Lactococcus lactis* subsp. *cremoris* AM2. *FEBS Lett.* **1999**, *445*, 321−324.

(8) Rutherfurd-Markwick, K. J. Food proteins as a source of bioactive peptides with diverse functions. *Br. J. Nutr.* **2012**, *108*, S149−S157.

(9) Korhonen, H.; Pihlanto, A. Bioactive peptides: production and functionality. *Int. Dairy J.* **2006**, *16*, 945−960.

(10) Hernández-Ledesma, B.; del Mar Contreras, M.; Recio, I. Antihypertensive peptides: production, bioavailability and incorporation into foods. *Adv. Colloid Interface Sci.* **2011**, *165*, 23−35.

(11) Nongonierma, A.; FitzGerald, R. J. Biofunctional properties of caseinophosphopeptides in the oral cavity. *Caries Res.* **2012**, *46*, 234−267.

(12) Power, O.; Jakeman, P.; FitzGerald, R. J. Antioxidative peptides: enzymatic production, in vitro and in vivo antioxidant activity and potential applications of milk-derived antioxidative peptides. *Amino Acids* **2013**, *44*, 797−820.

(13) Ricci-Cabello, I.; Herrera, M. O.; Artacho, R. Possible role of milk-derived bioactive peptides in the treatment and prevention of metabolic syndrome. *Nutr. Rev.* **2012**, *70*, 241−255.

(14) Adler-Nissen, J. *Enzymatic Hydrolysis of Food Proteins*; Elsevier Science Publishing: London, UK, 1986.

(15) Spellman, D.; O'Cuinn, G.; FitzGerald, R. J. Physicochemical and sensory characteristics of whey protein hydrolysates generated at different total solids levels. *J. Dairy Res.* **2005**, *72*, 138−143.

(16) Duvetter, T.; Sila, D. N.; Van Buggenhout, S.; Jolie, R.; Van Loey, A.; Hendrickx, M. Pectins in processed fruit and vegetables: part I—stability and catalytic activity of pectinases. *Compr. Rev. Food Sci. Food Saf.* **2009**, *8*, 75−85.

(17) Yemenicioğlu, A.; Özkan, M.; Cemeroğlu, B. Heat Inactivation kinetics of apple polyphenoloxidase and activation of its latent form. *J. Food Sci.* **1997**, *62*, 508−510.

(18) Gouzi, H.; Depagne, C.; Coradin, T. Kinetics and thermodynamics of the thermal inactivation of polyphenol oxidase in an aqueous extract from *Agaricus bisporus*. *J. Agric. Food Chem.* **2012**, *60*, 500−506.

(19) Forsyth, J. L.; Owusu Apenten, R. K.; Robinson, D. S. The thermostability of purified isoperoxidases from *Brassica oleracea* var. *gemmifera*. *Food Chem.* **1999**, *65*, 99−109.

(20) Dumitraşcu, L.; Stănciuc, N.; Stanciu, S.; Râpeanu, G. Thermal inactivation of lactoperoxidase in goat, sheep and bovine milk − a comparative kinetic and thermodynamic study. *J. Food Eng.* **2012**, *113*, 47−52.

(21) Nicoli, M. C.; Anese, M.; Parpinel, M. T.; Franceschi, S.; Lerici, C. R. Loss and/or formation of antioxidants during food processing and storage. *Cancer Lett.* **1997**, *114*, 71−74.

(22) You, L.; Zheng, L.; Regenstein, J. M.; Zhao, M.; Liu, D. Effect of thermal treatment on the characteristic properties of loach peptide. *Int. J. Food Sci. Technol.* **2012**, *47*, 2574−2581.

(23) Kilcawley, K. N.; Wilkinson, M. G.; Fox, P. F. Determination of key enzyme activities in commercial peptidase and lipase preparations from microbial or animal sources. *Enzyme Microb. Technol.* **2002**, *31*, 310−320.

(24) Smyth, M.; FitzGerald, R. J. Relationship between some characteristics of WPC hydrolysates and the enzyme complement in commercially available proteinase preparations. *Int. Dairy J.* **1998**, *8*, 819−827.

(25) Adjonu, R.; Doran, G.; Torley, P.; Agboola, S. Screening of whey protein isolate hydrolysates for their dual functionality: influence of heat pre-treatment and enzyme specificity. *Food Chem.* **2013**, *136*, 1435−1443.

(26) Ludikhuyze, L. R.; Claeys, W. L.; Hendrickx, M. E. Effect of temperature and/or pressure on lactoperoxidase activity in bovine milk and acid whey. *J. Dairy Res.* **2001**, *68*, 625−637.

(27) Mullally, M. M.; Meisel, H.; FitzGerald, R. J. Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine $\beta$-lactoglobulin. *FEBS Lett.* **1997**, *402*, 99−101.

(28) Fadiloğlu, S.; Erkmen, O.; Şekeroğlu, G. Thermal inactivation kinetics of alkaline phosphatase in buffer and milk. *J. Food Process. Preserv.* **2006**, *30*, 258−268.

(29) De Luis, R.; Pérez, M. D.; Sánchez, L.; Lavilla, M.; Calvo, M. Kinetic and thermodynamic parameters for heat denaturation of Cry1a(b) protein from transgenic maize (*Zea mays*). *J. Food Sci.* **2008**, *73*, C447−C451.

(30) Stănciuc, N.; Dumitrascu, L.; Râpeanu, G.; Stanciu, S. $\gamma$-Glutamyl transferase inactivation in milk and cream: a comparative kinetic study. *Innovative Food Sci. Emerging Technol.* **2011**, *12*, 56−61.

(31) Beliciu, C. M.; Moraru, C. I. The effect of protein concentration and heat treatment temperature on micellar casein−soy protein mixtures. *Food Hydrocolloids* **2011**, *25*, 1448−1460.
(32) Bryant, C. M.; McClements, D. J. Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends Food Sci. Technol.* **1998**, *9*, 143−151.
(33) O'Loughlin, I. B.; Murray, B. A.; Kelly, P. M.; FitzGerald, R. J.; Brodkorb, A. Enzymatic hydrolysis of heat-induced aggregates of whey protein isolate. *J. Agric. Food Chem.* **2012**, *60*, 4895−4904.
(34) Tseng, Y.-C.; Moreira, R.; Sun, X. Total frying-use time effects on soybean-oil deterioration and on tortilla chip quality. *Int. J. Food Sci. Technol.* **1996**, *31*, 287−294.
(35) Debnath, S.; Rastogi, N. K.; Krishna, A. G. G.; Lokesh, B. R. Effect of frying cycles on physical, chemical and heat transfer quality of rice bran oil during deep-fat frying of poori: an Indian traditional fried food. *Food Bioprod. Process.* **2012**, *90*, 249−256.
(36) Kilshaw, P. J.; Heppell, L. M.; Ford, J. E. Effects of heat treatment of cows' milk and whey on the nutritional quality and antigenic properties. *Arch. Dis. Child.* **1982**, *57*, 842−847.
(37) Tang, X.; Wu, Q.; Le, G.; Shi, Y. Effects of heat treatment on structural modification and in vivo antioxidant capacity of soy protein. *Nutrition* **2012**, *28*, 1180−1185.