



Continuous Monitoring of Enzymatic Whey Protein Hydrolysis. Correlation of Base Consumption with Soluble Nitrogen Content

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The optimization of enzymatic protein hydrolysis often represents a tedious task due to complicated analytical methods. The simplest system of continuous analysis consists of monitoring the base consumption during a pH-controlled reactor operation. However, there are other criteria commonly used for characterizing the extent of protein hydrolysis, comprising the degree of hydrolysis (DH) and the SN-TCA index, that is the fraction of nitrogen soluble in trichloroacetic acid under well-defined conditions. Taking the SN-TCA index, expressed in terms of the so-called fraction of non-protein nitrogen NPN, as an example, it is shown that these criteria may be correlated with base consumption. The advantages and limitations of this method are discussed.

NOTATION

a_i	Regression coefficients	c_{pb}^0	Initial concentration of peptide bonds (mol litre ⁻¹)
c_B	Concentration of a strong base used for pH-control (mol litre ⁻¹)	DH	Degree of hydrolysis (%)
c_C^-	Concentration of terminal carboxylate ions (mol litre ⁻¹)	E_o	Enzyme (protease) concentration (g litre ⁻¹)
c_C^N	Concentration of terminal protonated carboxyl groups (mol litre ⁻¹)	K_a^C	Acid dissociation constant of terminal carboxyl groups (mol litre ⁻¹)
c_H	Concentration of hydronium ions (mol litre ⁻¹)	K_a^N	Acid dissociation constant of terminal amino groups (mol litre ⁻¹)
c_N^+	Concentration of terminal protonated amino groups (mol litre ⁻¹)	M_{pb}	Apparent mean molar mass associated with a peptide bond (g mol ⁻¹)
c_N^-	Concentration of terminal deprotonated amino groups (mol litre ⁻¹)	m_p^0	Initial protein mass in the reactor (g)
		n_B	Number of moles of strong base added for pH-control (mol)
		n_{Bm}	Number of moles of strong base added per unit mass of protein present (μmol g ⁻¹)
		n_{pb}^0	Initial number of moles of peptide bonds (mol)

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does not exhibit any appreciable buffer capacity since the amino group of the peptide bonds does not accept a proton in the whole range of pH from 1 to 14 due to the partial double-bond character of the peptide bond. In addition, the low concentration of terminal amino and carboxyl groups initially present are characterized by pK_a values of about 3.1–3.6 and 7.5–7.8, respectively, at 25°C, whereas the pK_a values of the protein side chains deviating only slightly from those of the free amino acids.^{3,8} Protein hydrolysis leads to the liberation of pairs of terminal amino and carboxyl groups exhibiting approximately the same pK_a values as given above, and which are subject to dissociation equilibria in the aqueous reaction mixture (Fig. 1). Assuming the formation of peptides with charged terminal groups, no change in pH would be observed since the proton potentially liberated by peptide bond cleavage would stay associated with the amino group. In the pH range in which the amino group undergoes a dissociation reaction, the formation of a neutral amino group would liberate protons. In contrast, the terminal carboxyl group consumes protons by formation of a neutral species. Therefore, the number of moles of strong acid or base added per mole of peptide bonds cleaved in order to maintain a constant pH may be given in terms of molar fractions as:

$$x_B = x_N^n - x_C^n \quad (1)$$

Assuming a constant mean acid dissociation constant for both terminal groups:

$$K_a^N = \frac{c_N^n c_H}{c_N^+} \text{ for: } R-NH_3^+ \rightleftharpoons R-NH_2 + H^+ \quad (2)$$

$$K_a^C = \frac{c_C^- c_H}{c_C^n} \text{ for: } R-COOH \rightleftharpoons R-COO^- + H^+ \quad (3)$$

and defining c_{pb}^0 as the total concentration of peptide bonds which may be cleaved and X as the actual fraction of peptide bonds which have been cleaved, the global balance yields:

$$c_{pb}^0 X = c_N^n + c_N^+ = c_C^n + c_C^- \quad (4)$$

and

$$x_N^n + x_N^+ = x_C^n + x_C^- = 1 \quad (4a)$$

With this, the molar fractions of neutral species may be described as:

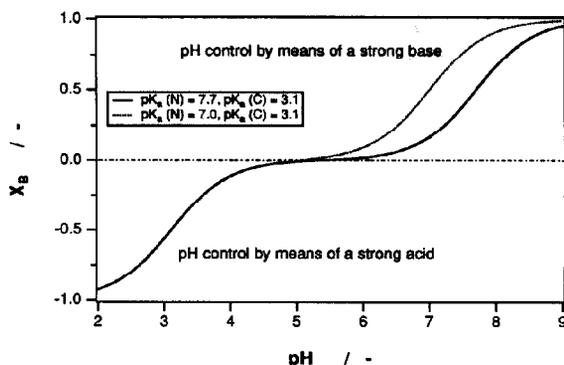


Fig. 2. Titratable fraction of peptide bonds cleaved.

$$x_N^n = \frac{c_N^n}{c_{pb}^0 X} = \frac{K_a^N}{1 + \frac{K_a^N}{c_H}} \quad (5)$$

$$x_C^n = \frac{c_C^n}{c_{pb}^0 X} = \frac{1}{1 + \frac{K_a^C}{c_H}} \quad (6)$$

With these results and remembering that $pH = -\log(c_H)$ and $pK = -\log(K)$, eqn (1) may be simplified to:

$$x_B = \frac{1}{1 + 10^{(pK_a^N - pH)}} - \frac{1}{1 + 10^{(pH - pK_a^C)}} \quad (7)$$

The function x_B , as illustrated in Fig. 2, represents the tritratable fraction of peptide bonds cleaved. A positive sign for x_B indicates that a base has to be added to maintain a constant pH and a negative sign indicates that an acid has to be added. Since an increase in temperature favours dissociation, the acid exponents of both terminal groups liberated upon protein hydrolysis are expected to be somewhat lower than at 25°C. Indeed, the acid exponent of the amino group changes from a mean value of 7.7 at 25°C to about 7.0 at 55°C, whereas the acid exponent of the carboxyl group changes only slightly with temperature.^{3,8} Figure 2 also indicates that the final pH for hydrolyses with a floating pH should converge at the isoelectric point, that is at a final pH of about 5.5 at 25°C or 5.0 at 55°C. At the isoelectric point $pI = \frac{1}{2}(pK_a^N + pK_a^C)$ the charges and the degree of protonation of the carboxyl and the amino group balance each

other ($x_N^n = x_C^n$). Hydrolysis at $\text{pH} = \text{pI}$, therefore, does not lead to a change in pH .

By definition, the degree of conversion (X) used above is identical with the degree of hydrolysis (DH) defined by Adler-Nissen.^{3,9} Re-defining the tritatable fraction x_B in terms of observable variables yields:

$$x_B = \frac{V_B c_B}{V_R c_{pb}^0 X} = x_N^n - x_C^n \quad (8)$$

The degree of hydrolysis may be correlated with the volumetric consumption of strong acid or base:

$$\text{DH} = X = \frac{1}{x_N^n - x_C^n} \frac{V_B c_B}{V_R c_{pb}^0} \quad (9)$$

A negative sign in eqn (9) again simply states that a strong acid has to be used for pH control. Equation (9) also indicates that the pH -stat method cannot be applied in the range of the isoelectric point — as discussed above. The number of moles of peptide bonds present in a reactor may be estimated from:

$$n_{pb}^0 = V_R c_{pb}^0 = \frac{m_P^0}{M_{pb}} \quad (10)$$

with the mean equivalent molar mass associated with a cleavable peptide bond (M_{pb}) of 113.6 g mol^{-1} for whey proteins.³

In the case of limited protein hydrolysis a fairly linear relationship is found for correlating the degree of hydrolysis with base consumption.³ This must not necessarily be the same for the relationship between the SN-TCA index and base consumption. The solubility may increase rapidly at low protein conversion, whereas at high conversion solubility may only change slowly due to the splitting of soluble peptides.

MATERIALS AND METHODS

Partially demineralized whey protein concentrate (WPC) with an average protein content of 22% (w/w) was employed as substrate. Hydrolysis was achieved with a commercial enzyme preparation of porcine pancreatic trypsin, PTN 6.0 S (Novo Nordisk, Denmark). Active site titration with *p*-nitrophenyl guanidinobenzoate according to the method of Chase and Shaw¹⁰ yielded a concentra-

tion of active enzyme of $10.2 \mu\text{mol g}^{-1}$ for this preparation.

All experiments were performed at a substrate concentration of 20% (w/w) WPC. Prior to starting the reaction, trypsin was solubilized in 1 mM HCl containing 5 mM CaCl_2 . The reaction was carried out in a jacketed stirred tank reactor, with a volume of 100 ml, at a constant temperature of either 55 or 60°C. The reactor was operated discontinuously. A small mechanical stirrer was operated at 1000 rev min^{-1} . The installation was equipped with a pH -meter (Metrohm 632, Switzerland) and a titrator-controller unit (Metrohm 614), enabling the reaction mixture to be maintained at a constant pH of 7.3 by feeding a 4 N KOH solution with an auto-burette (Metrohm 535). The volume of strong base added during the reaction was recorded on a PC equipped with a data acquisition board. The protein solution was treated for 5 min at 90°C prior to starting the reaction by addition of the enzyme. The total time required for this treatment at 90°C was about 13–14 min.

The SN-TCA index is expressed in terms of the fraction of soluble nitrogen (NPN: 'non-protein nitrogen'). The SN-TCA index was evaluated by treating samples of the reaction mixture with an aqueous solution of 13.6% (w/w) trichloroacetic acid. For the analysis of the NPN, both the original sample of the reactor contents and a sample of the supernatant after TCA treatment and centrifugation were analysed by the Kjeldahl method. The Kjeldahl digestion was carried out in a Büchi 402 rapid digestion unit (Flawil, CH) and a distillation unit (Büchi 322) equipped with an automatic control unit for titration (Büchi 343). The NPN values were calculated by dividing the soluble nitrogen concentration by the total nitrogen concentration.

RESULTS AND DISCUSSION

Figure 3 shows an example of a discontinuous reaction without pH control. The drop in pH is rather steep due to the poor buffering capacity of the proteins, followed by a slow descent at low pH . This slow descent may be explained both by the approach of the isoelectric point of the terminal groups liberated and by the low activity of trypsin in this range of pH .

The correspondence of the degree of protein hydrolysis expressed in terms of NPN and the

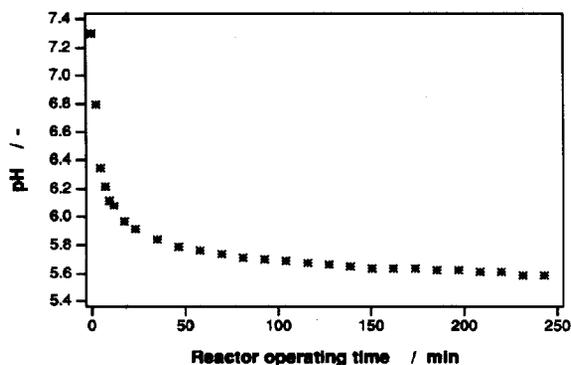


Fig. 3. An example of protein hydrolysis without pH control ($T_c = 55^\circ\text{C}$, $S_o = 20\%$ (w/w), $E_o/S_o = 0.01$).

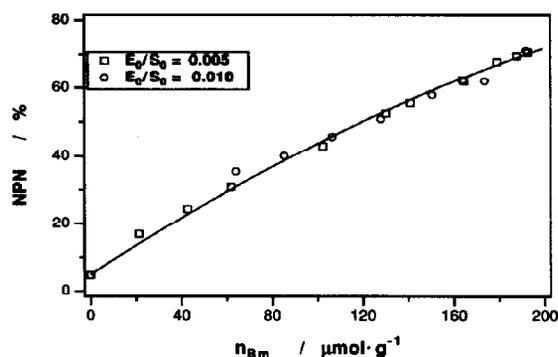


Fig. 4. Correlation of the soluble protein fraction expressed as NPN and the amount of strong base consumed per unit mass of protein ($T_c = 55^\circ\text{C}$, $S_o = 20\%$ (w/w), pH = 7.3).

mass-specific base consumption is given in Figs 4 and 5 for reaction temperatures of 55 and 60°C, respectively. These correlations were established at two different enzyme-substrate ratios.

The dependence of NPN on base consumption may conveniently be expressed in the form of polynomials:

$$\text{NPN} - \text{NPN}_0 = a_1 n_{Bm} - a_2 n_{Bm}^2 \quad (\%) \quad (11)$$

with

$$n_{Bm} = \frac{1}{m_p^o} V_B c_B = \frac{n_B}{m_p^o} \quad (\mu\text{mol g}^{-1}) \quad (11a)$$

where NPN_0 is the initial non-protein nitrogen fraction of the whey protein concentrate. The WPC employed had an initial NPN of 4.8%. The coefficients of the correlations are gathered in Table 1 for both reaction temperatures of 55 and

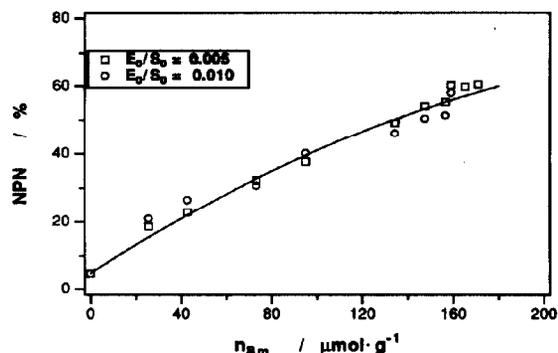


Fig. 5. Correlation of the soluble protein fraction expressed as NPN and the amount of strong base consumed per unit mass of protein ($T_c = 60^\circ\text{C}$, $S_o = 20\%$ (w/w), pH = 7.3).

Table 1. Regression coefficients for the correlation of NPN and base consumption

Temperature (°C)	a_1 (% g μmol^{-1})	a_2 (% g ² μmol^{-2})
55	0.44	-0.524×10^{-3}
60	0.42	-0.584×10^{-3}

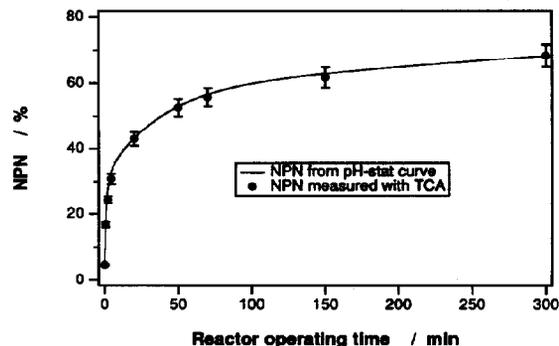


Fig. 6. Discontinuous hydrolysis reaction for testing the correlation between NPN and base consumption ($T_c = 55^\circ\text{C}$, $S_o = 20\%$ (w/w), $E_o/S_o = 0.005$, pH = 7.3).

60°C. As expected, the correlation between the soluble nitrogen content and the quality of base added per unit mass of whey protein is not linear because, as discussed earlier, the concentration of soluble peptides formed cannot be expected to be a linear function of peptide bonds cleaved.

An independent measurement at an operating temperature of 55°C is given in Fig. 6. Equation (11) together with the coefficients of Table 1 have been used for calculating the fraction of soluble

protein (shown as a continuous line in Fig. 6). Some parallel analyses of the fraction of soluble protein by means of the TCA method indicate a close agreement of both results.

CONCLUSION

The SN-TCA index, a criterion of practical importance for characterizing protein hydrolysates, has been correlated with the amount of base added for pH control. This allows the simple pH-stat procedure to be used for continuous monitoring of the degree of reaction. Thus, once calibrated under different reaction conditions, data about the reaction system may be readily obtained.¹¹ This method may be applied not only in this particular case, but also for analysing the hydrolysis of other proteins by means of other proteases, unless the pH optimum of the enzyme falls into the range of the isoelectric point of the dissociating groups liberated during hydrolysis.

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