# Correlation of base consumption with the degree of hydrolysis in enzymic protein hydrolysis

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Summary. It is fairly easy to control the enzymic hydrolysis of proteins in alkaline conditions by measuring the base consumption required to keep the pH constant in the reactor. Unfortunately, however, base consumption is not related in any simple way to the degree of hydrolysis reached at any given moment and to establish this relationship it is essential to find out the mean pK of the $\alpha$-amino groups released during the hydrolytic process. We have shown here that the correct mean pK value varies according to the pH of the working conditions and that the relationship between these values may depend upon the kind of protein and protease used. We have put forward a method for determining this relationship experimentally by using a given protein–protease system, consisting of an alkaline titration of the raw protein and when partially hydrolysed. We have tested the results predicted by our theoretical model by applying it to the hydrolysis of whey proteins with a bacterial protease from *Bacillus licheniformis* at 50 °C, pH 8·0. This model can easily be applied to any hydrolytic process involving the appearance of functional groups that are partially protonizable under the working conditions in question in order to follow the kinetics of the reaction via the consumption of the neutralizing agent required to keep pH constant.

Keywords: Protein hydrolysis, proteases, pK, degree of hydrolysis, milk protein.

Protein hydrolysates are widely used as food flavours and protein complements in energy-giving drinks, and also, quite importantly, in the preparation of enteral diets for children and sick adults. For these latter purposes the hydrolysates are generally derived from milk proteins (Chiang *et al*. 1982; Nakamura *et al*. 1993). The requirements demanded in the formulation of these hydrolysates, i.e. that they should not have a bitter taste, should be hypoallergenic, should be low in free amino acids and have a very controlled peptide size (Ney, 1979; Grimble *et al*. 1986; Otani *et al*. 1990), all demand that the reaction process be carefully monitored. To this end, the application of the pH-stat technique allows us to control the hydrolytic process fairly easily (Jacobsen *et al*. 1957). Nevertheless, the consumption of the agent used to maintain pH constant is not related in any simple way to the degree of hydrolysis

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Table 1. *pK values at 50 °C derived from Adler-Nissen (1986) by comparing base consumption and α-amino groups released during hydrolysis at two different pH values. The enzyme used was Alcalase 0.6L and substrate concentration was 50 g/l*

| Substrate | $pH_1$ | $pH_2$ | pK |
|---|---|---|---|
| Soyabean protein | 6·5 | 7·0 | 7·05 |
| | 6·5 | 7·5 | 7·10 |
| | 6·5 | 8·0 | 7·14 |
| | 6·5 | 9·5 | 7·21† |
| | 7·0 | 7·5 | 7·15 |
| | 7·0 | 8·0 | 7·19 |
| | 7·0 | 9·5 | 7·27† |
| | 7·5 | 8·0 | 7·25 |
| | 7·5 | 9·5 | 7·36† |
| | 8·0 | 9·5 | 7·48† |
| Casein | 7·0 | 7·5 | 6·85 |

† Values calculated by authors using Adler-Nissen's (1986) method and experimental results.

reached at any moment and to establish this relationship it is essential to know the pK of the α-amino groups released during the hydrolytic process.

The pK value was first determined by Adler-Nissen (1986) by comparing the base consumption with the analysis of the free α-amino groups released during hydrolysis. Most research workers in this field have since used these results (Antila, 1988; González-Tello *et al.* 1994; Margot *et al.* 1994; Camacho *et al.* 1998; Dzwolak & Ziajka, 1999). To determine the pK at 50 °C Adler-Nissen made five experiments with a soyabean protein concentrate at pH 6·5, 7·0, 7·5, 8·0 and 9·5 and two experiments with casein at pH 7·0 and 7·5, all using the protease Alcalase 0.6L and a solution of 50 g protein/l. He followed the progress of hydrolysis with reference to the addition of the base necessary to keep pH constant and an analysis of the free α-amino groups using the trinitrobenzene sulphonic acid (TNBS) method (Adler-Nissen, 1979). He then developed a method for determining pK based upon a comparison between two experiments made at different pH values, which gave rise to the equation

$$pK = pH_2 + \log_{10}(b_{pH_1} - b_{pH_2}) - \log_{10}(b_{pH_2} 10^{pH_2 - pH_1} - b_{pH_1}), \quad (1)$$

where $b_{pH}$ represents the relationship between the equivalents determined by the TNBS method and base consumption, which, according to his results, seems to stay constant until practically 20% hydrolysis. The results obtained by this method are set out in Table 1, where we also include the results at pH = 9·5, which Adler-Nissen himself did not use.

Using values excluding those from the experiments at pH 9·5 Adler-Nissen came to the conclusion that the differences were insignificant, since from a theoretical point of view the pK values might be expected to be practically identical, and thus he decided to average them out, and proposed a value of pK = 7·1 at 50 °C, in which he included both substrates used (soyabean protein, mean pK = 7·15 and casein, mean pK = 6·85). Although he mentions that his results at pH 9·5 are somewhat higher he puts this down to the possible effects of amino acid side-chains or to the fact that the pK is a mean of the values of very different terminal α-amino groups. It is quite clear, however, that Adler-Nissen's pK values increase concomitantly with the pH of the experiment in question and that the difference between the two substrates used is considerable.

Other authors (Margot *et al.* 1994) have tried to relate base consumption with other factors pertaining to the conditions of the hydrolytic reaction, such as soluble

nitrogen, i.e. nitrogen that remains dissolved in an aqueous trichloroacetic acid solution under normalized conditions.

Our aim in this paper is to show by a theoretical analysis of the process that the correct pK value, which must be a mean of the values of the different $\alpha$-amino groups released during the hydrolytic process, changes according to the pH of the experimental conditions. We then propose a practical method to determine the relationship between pK and pH for any given protein–protease system.

## THEORETICAL ASPECTS

*Nomenclature*

| | |
|---|---|
| a | Slope of the linear relationship between pH and pK, eqn 19 (−) |
| B | Base added (mol) |
| $b_{pH}$ | Relationship derived by Adler-Nissen between the equivalents determined by the TNBS method and base consumption (−) |
| $b_s$ | Base consumption in the titration for original protein (mol) |
| $b_x$ | Base consumption in the titration for partially hydrolysed protein (mol) |
| $C_B$ | Concentration of base (M) |
| $C_F$ | Overall concentration of both unprotonated and protonated groups (M) |
| $C_i$ | Concentration of particular $\alpha$-amino group released (M) |
| $C_T$ | Overall concentration of $\alpha$-amino groups (M) |
| F | Unprotonated group, lateral and terminal |
| $FH^+$ | Protonated group, lateral and terminal |
| $h_T$ | Peptide bonds per gram of protein (mol/g) |
| K | Equilibrium constant for the deprotonization of the amide group (mol) |
| k | Coefficient calculated by Adler-Nissen between the $\alpha$-amino groups determined by the TNBS method and the degree of hydrolysis (−) |
| Mp | Mass of protein (g) |
| pI | Ionic product of water (−) |
| $P–NH_2$ | Unprotonated terminal amino group |
| $P–NH_3^+$ | Protonated terminal amino group |
| $S_0$ | Initial protein concentration (g/l) |
| $V_B$ | Volume of base (litres) |
| $V_0$ | Initial volume of solution (litres) |
| WPC | Whey protein concentrate |
| x | Degree of hydrolysis, expressed as a fraction (−) |
| $\beta$ | Value of $1/(1+10^{pKo-pHo})$ at pH in which titration begins (−) |
| $\gamma$ | Activity coefficient (−) |
| $\mu$ | Centre of the distribution eqn (17) |
| $\sigma$ | Standard deviation eqn (17) |

*Relationship between pK and pH*

When an amide bond is hydrolysed under alkaline conditions, $7 < pH < 10$:

$$P–CO–NH–P + H_2O \rightarrow P–COOH + P–NH_2, \quad (2)$$

the terminal carboxyl group is completely dissociated

$$P–COOH \rightarrow P–COO^- + H^+, \quad (3)$$

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and the protons thus formed are distributed according to the protonization equilibrium of the $\alpha$-amino released:

$$P\text{–}NH_3^+ \Leftrightarrow P\text{–}NH_2 + H^+, \tag{4}$$

i.e. for each mol of hydrolysed amide bonds there appears one mol of monovalent anions, P–$COO^-$, and one mol of monovalent cations distributed between both species: P–$NH_3^+$ and $H^+$. The base added to keep pH constant only neutralizes the protons, which are then replaced by the cation belonging to the base, and thus the mols of the added base are equivalent to the protons generated by the hydrolytic process, which are only a fraction of the amide bonds hydrolysed.

The equilibrium of stage (4), which can be taken as being instantaneous, involving as it does the exchange of only one proton, allows us to calculate the fraction of the hydrolysed amide bonds that must be neutralized by the base to keep pH constant, and so to relate base consumption with the degree of hydrolysis reached at that moment; fulfilling thus

$$\frac{[P\text{–}NH_2][H^+]}{[P\text{–}NH_3^+]} = K, \tag{5}$$

where K is the equilibrium constant for the deprotonization of the amide group.

If we introduce into eqn (5) the values of

$$K = 10^{-pK} \quad [H^+] = 10^{-pH}, \tag{6}$$

we get

$$\frac{[P\text{–}NH_2]}{[P\text{–}NH_3^+]} = 10^{pH-pK}, \tag{7}$$

and the fraction in question will be

$$\frac{[P\text{–}NH_2]}{[P\text{–}NH_2]+[P\text{–}NH_3^+]} = \frac{10^{pH-pK}}{1+10^{pH-pK}}, \tag{8}$$

and thus the relationship between the mols of peptide bonds hydrolysed and those of the added base will be

$$S_0 h_T dx = \frac{1+10^{pH-pK}}{10^{pH-pK}}\, dB = (1+10^{pK-pH})dB, \tag{9}$$

where $S_0$ represents g protein/l, $h_T$ the equivalents of peptide bonds per gram of protein, x the degree of hydrolysis of the hydrolysate used.

That is to say, to calculate the degree of hydrolysis reached it is essential to know the relevant pK value. Nevertheless, eqn (7) must involve an average of the different $\alpha$-amino groups released during hydrolysis, which are partially protonizable at the pH in question:

$$P_iNH_3^+ \Leftrightarrow P_iNH_2 + H^+. \tag{10}$$

For equilibrium (10) eqn (7) should be written as

$$\frac{[P_iNH_2]}{[P_iNH_3^+]} = 10^{pH-pKi}, \tag{11}$$

with which, if we denote the mol/l, either free or protonated, released of $P_i$–$NH_2$ as $C_i$ then the terms of

$$[P_iNH_2]+[P_iNH_3^+] = [P_iNH_3^+](1+10^{pH-pKi}) = C_i, \tag{12}$$

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are fulfilled and thus the concentration of the protonated form can be expressed as

$$[P_iNH_3^+] = \frac{C_i}{(1+10^{pH-pKi})}. \tag{13}$$

If we sum eqn (13) for all the groups released

$$[P\text{–}NH_3^+] = \sum_i [P_iNH_3^+] = \sum_i \frac{C_i}{(1+10^{pH-pKi})}, \tag{14}$$

given that the concentration of the protonated form of all the $\alpha$-amino groups released, using the mean pK defined by eqn (7), is

$$[P\text{–}NH_3^+] = \frac{C_T}{(1+10^{pH-pK})}, \tag{15}$$

where $C_T$ is the molarity of the hydrolysed amide bonds, a comparison of eqns (14) and (15) shows that the mean value for the pK can be expressed as

$$pK = pH - \log_{10}\left( \frac{1}{\sum_i \frac{C_i/C_T}{1+10^{pH-pKi}}} - 1 \right), \tag{16}$$

from which equation we can see that the correct mean pK value may depend upon the pH of the experimental conditions.

*Distribution of the $pK_i$*

During the enzymic hydrolysis of a protein with a specific enzyme it is fair to presume that this enzyme particularly catalyses the hydrolysis of peptide bonds involving one particular amino acid or a group of similar amino acids. But nevertheless, the pK of the $\alpha$-amino group thus released will be affected by the nature of the nearest amino acids, which will cause a variation, within a certain range, in the $pK_i$ values of the $\alpha$-amino groups released. If we presume that there is normal distribution centred at $\mu$ with a standard deviation of $\sigma$, the fraction of $\alpha$-amino groups released with a pK value of between pK and pK + dpK will be

$$\frac{1}{(2\pi\sigma^2)^{1/2}} \exp\left( -\frac{(pK-\mu)^2}{2\sigma^2} \right) dpK. \tag{17}$$

For this continuous distribution the summation in eqn (16) must be transformed into an integral

$$\sum_i \frac{C_i/C_T}{1+10^{pH-pK_i}} = \int_0^\infty \frac{\exp\left( -\frac{(pK-\mu)^2}{2\sigma^2} \right)}{(2\pi\sigma^2)^{1/2}(1+10^{pH-pK})} dpK, \tag{18}$$

which can be calculated for any pH value, for any given values of the two parameters that characterize the distribution: $\mu$ and $\sigma$; in accordance with eqn (14) the expression (18) represents the protonated fraction of the $\alpha$-amino groups released during hydrolysis. Thus, we can determine the mean pK value for each pH by introducing eqn (18) in eqn (16) and solving the integration by MatLab 5.1 (The Mathworks Inc., Natick, MA 01760, USA). As an example, the results obtained for

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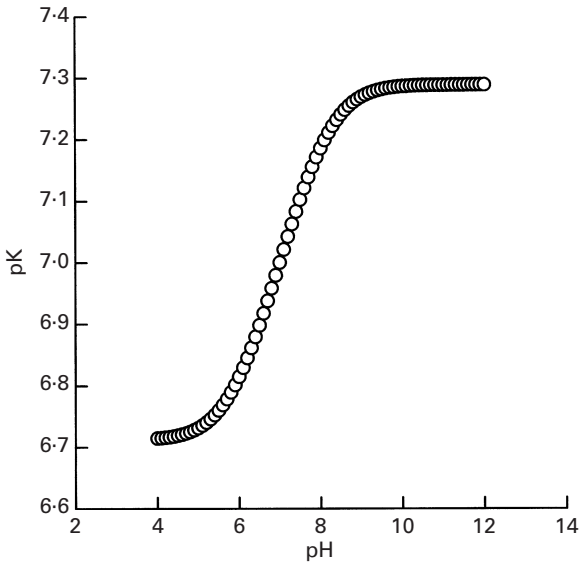


Fig. 1. Variation of mean pK *v.* pH. Simulation of the proposed method for a normal distribution of the $pK_i$ values centred at $\mu = 7{\cdot}0$ with a standard deviation of $\sigma = 0{\cdot}5$.

a distribution of $\mu = 7{\cdot}0$ and $\sigma = 0{\cdot}5$ are plotted in Fig. 1. If we are interested in the pH range between those which determine a higher protonated fraction at pH 0·95 and a lower one at 0·05, by using eqn (17) it is possible to determine this range, the size of which depends wholly upon the width of the distribution, $\sigma$, and is centred in the middle of the distribution, $\mu$. The values obtained through this calculation are set out in Table 2. Outside this range it may be accepted that all the $\alpha$-amino groups released are either protonated (low pH values) or deprotonated (high pH values). As mentioned above, by using eqn (16) we can arrive at the correct mean pK value within this range.

Some of the results obtained via this procedure can be seen in Fig. 2, for distributions of $\mu = 7{\cdot}5$ and $\sigma$ values from 0·2 to 1·2. It can be seen in this figure that the pK values change linearly *v.* pH within the range in question. The results obtained for $\mu = 7{\cdot}0$ and $\sigma$ values from 0·2 to 1·2 are set out for example's sake in Table 3. The slopes of the lines and the regression coefficients, $r^2$, turn out to be independent of the value of $\mu$, and, as can be seen, the fitting is satisfactory in all cases. Given that all the lines pass through the point $(\mu, \mu)$, the intersect at the origin is determined by the values of $\mu$ and of the slope, in such a way that in the range in question the relationship between the correct mean pK value and pH are given by

$$\mathrm{pK} = \mu + a(\mathrm{pH} - \mu). \tag{19}$$

The value of parameter $a$ depends exclusively upon the standard deviation in the distribution. In Fig. 3 we show this dependence, adjusted to the expression, in the considered interval.

$$a = \frac{0{\cdot}956\sigma^2}{1 + 1{\cdot}353\sigma^{1{\cdot}872}}. \tag{20}$$

In fact, if we accept the $pK_i$ values of the $\alpha$-amino groups released, characterized by their mean value, $\mu$, and their standard deviation, $\sigma$, the mean pK values calculated from eqn (16) vary within the pH range assayed (from the pH at which

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Table 2. *pH range used to calculate pK values by eqns (16) and (18), see text. This interval determines a variation of the protonated fraction of α-amino groups from 0·95 to 0·05, for a normal distribution centred at μ with a standard deviation of σ*

| $\sigma$ | pH intervals |
|---|---|
| 0·2 | $\mu \pm 6{\cdot}6\sigma$ |
| 0·4 | $\mu \pm 3{\cdot}6\sigma$ |
| 0·6 | $\mu \pm 2{\cdot}7\sigma$ |
| 0·8 | $\mu \pm 2{\cdot}3\sigma$ |
| 1·0 | $\mu \pm 2{\cdot}1\sigma$ |
| 1·2 | $\mu \pm 2{\cdot}0\sigma$ |

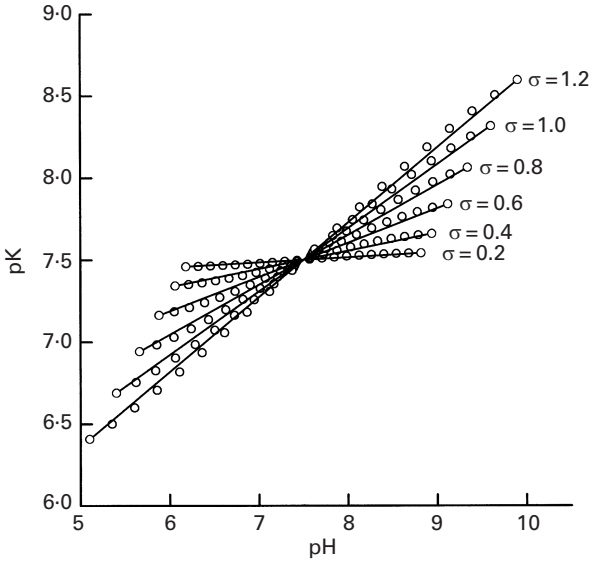


Fig. 2. Variation of mean pK *v.* pH in the linear range, see Table 2. Simulation of the proposed method for a normal distribution of the $pK_i$ values centred at $\mu = 7{\cdot}5$ with different values of the standard deviation $\sigma$.

more than 95 % of the α-amino groups released are protonized to the pH at which less than 5 % are protonized) in a straight line, as indicated by

$$\mathrm{pK} = \mu + a(\mathrm{pH} - \mu) = \mu + \frac{0{\cdot}956\sigma^2}{1 + 1{\cdot}353\sigma^{1{\cdot}872}}(\mathrm{pH} - \mu). \tag{21}$$

*Determination of the mean pK*

To determine the correct pK values for the hydrolysis of any given protein with any given protease we may use the following procedure based upon a titration and comparison within the alkaline range of the raw protein solution and another solution of equal concentration of the partially hydrolysed protein.

When we titrate a solution containing protonizable groups using a base, and include all these groups in F,

$$\mathrm{FH^+} \Leftrightarrow \mathrm{F} + \mathrm{H^+}, \tag{22}$$

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Table 3. *Linear regression parameters, at different values of the standard deviation σ for a normal distribution centred at μ = 7·0, of pK values* v. *pH in the linear range* (*see Table 2*)

| σ | Slope | Intersect | $r^2$ | (μ-intersect)/μ |
|---|---|---|---|---|
| 0·2 | 0·0357 | 6·7504 | 0·9855 | 0·0357 |
| 0·4 | 0·1231 | 6·1387 | 0·9912 | 0·1230 |
| 0·6 | 0·2265 | 5·4146 | 0·9952 | 0·2265 |
| 0·8 | 0·3235 | 4·7355 | 0·9973 | 0·3235 |
| 1·0 | 0·4061 | 4·1571 | 0·9983 | 0·4061 |
| 1·2 | 0·4741 | 3·6813 | 0·9989 | 0·4741 |

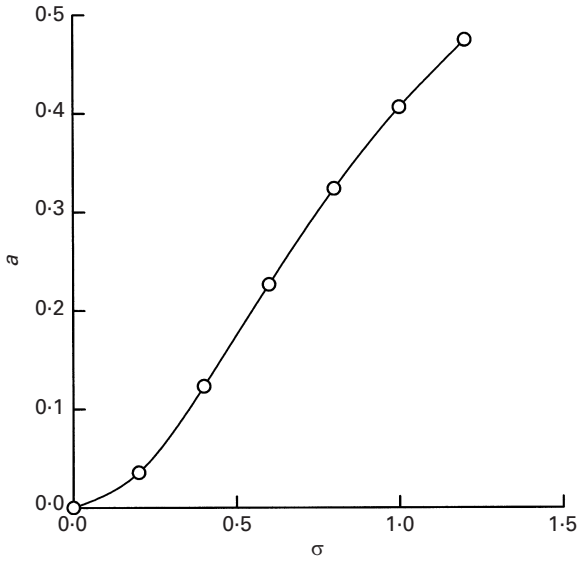


Fig. 3. Variation of the slope of the equation $pK = \mu + a\ (pH - \mu)$ *v.* standard deviation $\sigma$.

the equilibrium will determine the variation in pH *v.* the quantity of base added. This equilibrium can be expressed as

$$\frac{[F]}{[FH^+]} = 10^{pH-pK}, \tag{23}$$

in which the correct mean pK value is given by eqn (16).
By applying eqn (23) at the start of the titration, we get

$$\frac{[F]_0}{[FH^+]_0} = 10^{pH_0-pK_0}, \tag{24}$$

and thus

$$[FH^+]_0 = \frac{C_F}{1+10^{pH_0-pK_0}}, \tag{25}$$

where $C_F$ is the overall concentration of F, both in free and protonated form.

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When we add a volume of base, $V_B$, at a concentration of $C_B$ to an initial volume of solution, $V_0$, the $OH^-$ will be used up partially in neutralizing protons and shifting the equilibrium (22) and the rest will remain free to modify the pH of the solution. The terms of eqn (23) must still be fulfilled, although the mean pK value will depend upon that of the new pH:

$$\frac{V_0[F]_0 + V_B C_B - ((V_0+V_B)10^{-pOH} - V_0 10^{-pOH_0})}{V_0[FH^+]_0 - V_B C_B + ((V_0+V_B)10^{-pOH} - V_0 10^{-pOH_0})} = 10^{pH-pK}, \tag{26}$$

and on introducing

$$b = V_B C_B/V_0 - ((1+V_B/V_0)10^{pH-pI} - 10^{pH_0-pI}), \tag{27}$$

that represents the base consumption used in shifting the equilibrium (22) and where pI is the ion product of the water at the temperature in question, eqn (26) takes the form

$$\frac{[F]_0 + b}{[FH^+]_0 - b} = 10^{pH-pK}, \tag{28}$$

If we then substitute eqns (24) and (25) into eqn (28) we get

$$\frac{C_F 10^{pH_0-pK_0}}{1+10^{pH_0-pK_0}} + b = \frac{C_F 10^{pH-pK}}{1+10^{pH_0-pK_0}} - b10^{pH-pK}, \tag{29}$$

which can be reordered into

$$b = C_F\left(\frac{1}{1+10^{pK-pH}} - \frac{1}{1+10^{pK_0-pH_0}}\right), \tag{30}$$

and if we accept that the mean pK can be expressed via eqn (21) the exponents in the second terms of the denominators of both fractions will be given by

$$pK - pH = (1-a)(\mu - pH) = \left(1 - \frac{0{\cdot}956\ \sigma^2}{1+1{\cdot}353\ \sigma^{1{\cdot}872}}\right)(\mu - pH), \tag{31}$$

From the experimental data (b, pH), eqns (30) and (31) can be used to obtain, by non-linear regression, the values for the parameters $C_F$, $\mu$ and $a$, and thus $\sigma$.

When a solution of the original protein is titrated by adding a base in the alkaline range, beginning with an initial pH, $pH_0$, the equivalents/l of base used up in the equilibrium shifting of protonization, calculated via eqn (27), $b_s$, are related logically to the pH reached. Nevertheless, this case involves the intervention of all the protonizable groups of the protein, whether terminal or not, other than those $\alpha$-amino groups that are released during hydrolysis. If for the same starting concentration and pH we titrate a partially hydrolysed solution of the protein, the equivalents/l of base used up, $b_x$, to reach any given pH will be determined by the protonizable groups existing in the initial protein plus those which have appeared as a consequence of hydrolysis. That is to say, $b_x - b_s$ corresponds to the protonizable groups which appear during the hydrolytic process, and the values of the parameters obtained by non-linear regression of eqns (30) and (31), with $b = b_x - b_s$, should correspond to the overall concentration of the $\alpha$-amino groups released during hydrolysis, $C_F$, and $\mu$ and $\sigma$ the parameters of normal distribution, which intervene in the expression for the variation in the mean pK *v.* the experimental pH, eqn (21). $C_F$ can be estimated as

$$C_F = S_0 h_T x. \tag{32}$$

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If the titration of the hydrolysed and non-hydrolysed protein is made until sufficiently alkaline conditions occur for all of the $\alpha$-amino groups released during hydrolysis to be deprotonated, then according to eqn (30) we should get a maximum value for $b_x - b_s$

$$(b_x - b_s)_{\text{maximum}} = C_F \left( 1 - \frac{1}{1 + 10^{pK_0 - pH_0}} \right) = C_F(1 - \beta). \tag{33}$$

in which the second term in brackets, which remains constant ($\beta$), corresponds to the fraction of the $\alpha$-amino groups released, which were already deprotonated at the initial pH of the titration, according to eqns (30) and (33). By dividing the values of $b_x$–$b_s$ by their maximum value we get

$$\frac{b_x - b_s}{(b_x - b_s)_{\text{maximum}}} = \frac{\dfrac{1}{1 + 10^{pK - pH}} - \beta}{1 - \beta}, \tag{34}$$

which should be independent of the degree of hydrolysis if the correct mean pK value does not change with hydrolysis.

The above procedure implies the hypothesis that the $pK_i$ of the amino acid side-chains are not modified by hydrolysis to any great extent.

Another factor that might also exert some influence is that eqn (7) must be written as

$$\frac{[P\text{–}NH_2]\,\gamma_1}{[P\text{–}NH_3^+]\,\gamma_2} = 10^{pH - pK}, \tag{35}$$

where $\gamma_1$ is the coefficient of activity of the free species, P–$NH_2$, and $\gamma_2$ the coefficient of activity of the protonated species. It should be borne in mind that the pH electrode measures the activity of $H^+$. If the effects of both coefficients of activity do not completely balance each other out, both, corresponding as they do to charged species, will fundamentally be a function of the ionic strength of the solution, which alters *v.* protein concentration and also pH (which determines the total charge of the species in question), and even with the addition of the base required to maintain pH constant. Thus, we can observe any possible influence of the coefficients of activity by intentionally modifying the ionic strength of the solution, by diluting it or adding a saline solution for example, and checking to see whether pH changes to any appreciable degree.

## Materials and methods

A commercial whey protein concentrate, WPC (Milei, 70191 Stuttgart, Germany) was used, containing 767 g protein/kg, 42·6 g moisture/kg, 2·5 g lactose/kg and 29·8 g ash/kg. The $h_T$ value, equivalents of peptide bonds per kg of protein, for this substrate was 8·8 (Novo Industries, 1980).

The enzyme used was Protex 6L, EC 3.4.21.62 (Genencor, Rochester, NY 14168, USA), a mainly alkaline bacterial protease obtained from a selected strain of *Bacillus licheniformis*. This enzyme presents maximum activity-stability at 50 °C and pH 8·0.

Titration and hydrolysis were carried out in a well-stirred temperature controlled batch reactor, capacity 1500 ml, connected to an automatic titrator and pH-controller Titrino 718 (Metrohm, CH-9100 Herisau, Switzerland) including a 2M-NaOH reservoir. The device has the following absolute errors in measurement: pH 0·003, 0·1 °C and 0·001 ml.

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Solutions of 10 g WPC/l were prepared by reconstituting WPC powder with Milli-Q water preheated at the experimental temperature of 50 °C and mixing with a stirrer until completely dissolved. The initial pH of the raw protein solution (6·05) was adjusted to 8·0, the protease was added (enzyme:substrate ratio = 0·005, reaction time < 20 min) and the hydrolysis process was monitored by the pH-stat technique. The equation derived by Adler-Nissen (1986), $x = (B\,(1+10^{pK-pH}))/(M_p\,h_T)$, was used with a pK value of 7·1 to calculate the base consumption corresponding to an established degree of hydrolysis.

When the calculated mols of base (x = 0·0, B = 0·00 mmol; x = 0·1, B = 11·68 mmol; x = 0·2, B = 23·36 mmol) had been consumed and thus the desired extent of the hydrolysis was reached, pH was rapidly decreased by HCl 35 %. Then titration from pH 6·5 to 10·0 was carried out (titration time < 5 min) recording both agent titration volume and pH. All experiments were duplicated. We used eqn (27) to determine the equivalents of titrant agent. The value for pI at 50 °C is 13·275.

To determine of influence of the activity coefficients in eqn (35) two different series of experiments were made. In the first series, ionic strength was modified by diluting a solution of 150 g protein/l to 100 g/l and to 50 g/l. In the second series, ionic strength was modified by adding consecutively 0·75, 6·75 and 67·5 ml 2 M-KCl to 1·5 litres of 50 g protein/l solution. In each case, changes in pH were studied.

SigmaPlot 4.0 (SPSS Inc., Chicago, IL 60606, USA) and MatLab 5.1 (The Mathworks Inc., Natick, MA 01760, USA) software packages were used for data analysis.

## RESULTS AND DISCUSSION

No changes in pH where observed when modifying ionic strength by dilution or addition of KCl solution. This result shows that, in practice, the activity coefficients in eqn (35) cancel each other.

The values of b, calculated via eqn (27), *v.* the pH for the raw protein and the hydrolysates deriving from 10 % and 20 % hydrolysis, calculated with a pK of 7·1, are shown in Fig. 4. The points corresponding to duplicated experiments are plotted in each series. Fig. 5 shows the values of $b_x - b_s$ *v.* pH for both degrees of hydrolysis, where it can be seen that these values reach a maximum, almost constant, value between pH 9·5 and 10. If we average out the results within this latter pH range we get

$$10\,\%\ \text{hydrolysis} \quad (b_x - b_s)_{\text{maximum}} = 0{\cdot}0063 \pm 0{\cdot}0002\ \text{M}.$$

$$20\,\%\ \text{hydrolysis} \quad (b_x - b_s)_{\text{maximum}} = 0{\cdot}0115 \pm 0{\cdot}0002\ \text{M}.$$

Fig. 6 is based upon these results in accordance with eqn (34), in which it can be seen that the results corresponding to both degrees of hydrolysis coincide and form a single curve. This implies that the mean pK value does not depend upon the degree of hydrolysis, at least between 0 % and 20 %.

If we substitute eqn (31) into eqn (34) and adjust the results of both experiments by non-linear regression via SigmaPlot (SPSS Inc.), we get

$$\mu = 6{\cdot}90 \pm 0{\cdot}05 \qquad a = 0{\cdot}45 \pm 0{\cdot}02 \qquad \beta = 0{\cdot}37 \pm 0{\cdot}02$$

with a good coefficient of determination (0·990), as can be seen in Fig. 6, where the curve predicted for the model with these values for the parameters is also shown. With the value of $a$ we can calculate the standard deviation in the distribution according to eqn (20): $\sigma = 1{\cdot}12$.

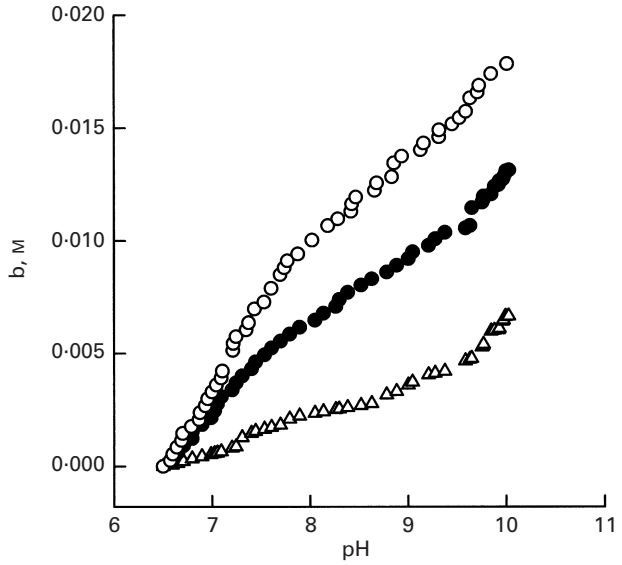


Fig. 4. Base consumption (b) in the titration from pH 6·5 to pH 10·0 of Δ, raw protein and hydrolysed protein at two different degrees of hydrolysis; ●, x = 0·1 and ○, x = 0·2.

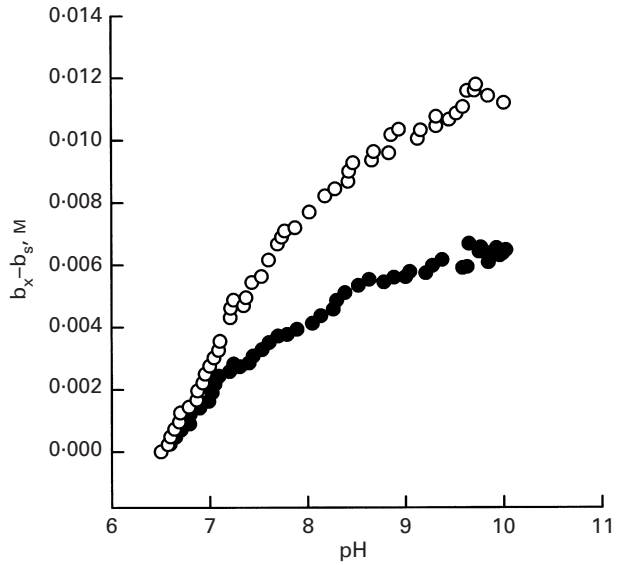


Fig. 5. Titration of α-amino groups released at two different degrees of hydrolysis; ● x = 0·1, ○, x = 0·2. Difference between base consumption for hydrolysed and native protein ($b_x - b_s$).

Thus, in accordance with our hypotheses, for the substrate–enzyme system in question the relationship between the correct mean pK and the experimental pH will be given by eqn (21):

$$\mathrm{pK} = 3{\cdot}8 + 0{\cdot}45\ \mathrm{pH}, \tag{36}$$

which, on being applied to pH 8, gives us a pK value of 7·4. These results are independent of the hypotheses set out in the Theoretical Aspects – *Distribution of the* $pK_i$ above, except for the fact that a linear relationship should exist between the

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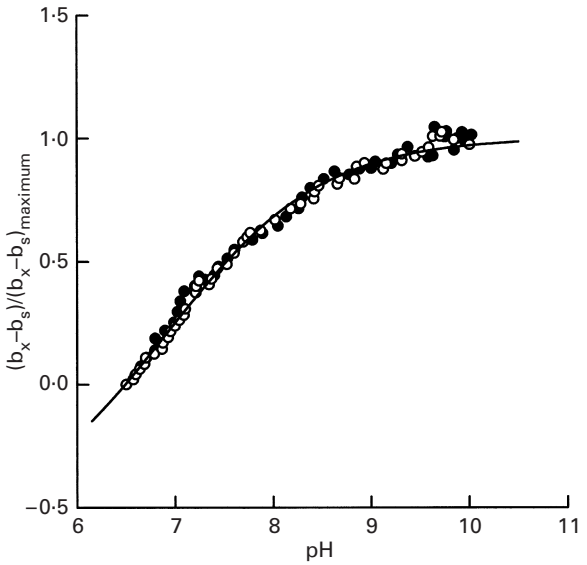


Fig. 6. Dimensionless base consumption $(b_x-b_s)/(b_x-b_s)_{max}$ *v.* pH. Results fitted to eqn (34). ●, x = 0·1; ○, x = 0·2; — eqn (34).

correct mean pK and pH. Nevertheless, the way it is dealt with in the Theoretical Aspects section leads us to a physical interpretation of the two parameters in this linear relationship.

If we correct the degrees of hydrolysis (which were calculated before with a pK of 7·1) to agree with this new value, we get

$$x_1 = 0{\cdot}10\frac{1+10^{7{\cdot}4-8}}{1+10^{7{\cdot}1-8}} = 0{\cdot}11,$$

$$x_2 = 0{\cdot}20\frac{1+10^{7{\cdot}4-8}}{1+10^{7{\cdot}1-8}} = 0{\cdot}22,$$

These results allow us to calculate the total concentration of $\alpha$-amino groups released during hydrolysis in both experiments via eqn (32):

$$C_{F1} = (10)\ (0{\cdot}0088)\ (0{\cdot}11) = 0{\cdot}0097 \text{ mol},$$

$$C_{F2} = (10)\ (0{\cdot}0088)\ (0{\cdot}22) = 0{\cdot}0194 \text{ mol},$$

and compare them with those obtained via eqn (33), in accordance with the model developed here:

$$C_{F1} = (b_{x1}-b_s)_{maximum}/(1-\beta) = 0{\cdot}0100 \text{ mol},$$

$$C_{F2} = (b_{x2}-b_s)_{maximum}/(1-\beta) = 0{\cdot}0183 \text{ mol},$$

which, as can be seen, agree satisfactorily.

The theoretical analysis made prior to the experiments indicated that the mean pK value may change with the experimental pH, just as Adler-Nissen's results really indicate (Table 1). However this author assumed that the pK does not change with the pH and thus he proposed a method for determining pK by comparing the results obtained with two different pH values. This method cannot be considered valid as is shown above. In fact, his results can be reinterpreted in the following way:

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Table 4. *pK values at 50 °C calculated by using Adler-Nissen's (1986) results. The enzyme used was Alcalase 0.6L and substrate concentration was 50 g/l*

| Substrate | pH | $b_{pH}$ | pK |
|---|---|---|---|
| Soyabean isolate | 6·5 | 5·93 | 7·21 |
| | 7·0 | 2·767 | 7·27 |
| | 7·5 | 1·663 | 7·35 |
| | 8·0 | 1·254 | 7·47 |
| | 9·5 | 0·971 | — |
| Casein | 7·0 | 2·732 | 7·21 |
| | 7·5 | 1·956 | 7·45 |

Using the coefficients, k, calculated by Adler-Nissen (1986) between the $\alpha$-amino groups determined by the TNBS method and the degree of hydrolysis, we can arrive at the pK values for each pH via

$$b_{pH} = k(1 + 10^{pK-pH}), \tag{37}$$

i.e.

$$pK = pH + \log_{10}\left(\frac{b_{pH}}{k} - 1\right), \tag{38}$$

where k = 0·970 for the soyabean protein concentrate and 1·039 for casein. The values thus obtained are set out in Table 4, except for those at pH 9·5 because at this pH the value of $b_{pH}$ was 0·971, i.e. practically the same as k, and thus all the $\alpha$-amino groups are practically free. That is to say that in this case base consumption gives the degree of hydrolysis directly, thus confirming Adler-Nissen's value for k.

From these results by linear regression we get the equations:

$$\text{soyabean protein isolate } pK = 6{\cdot}08 + 0{\cdot}17\ pH,$$
$$\text{casein } pK = 3{\cdot}85 + 0{\cdot}48\ pH,$$

which are similar to our eqn (36), especially in the second case, which is also a milk protein.

According to our results, it has been proved that the correct pK of the $\alpha$-amino groups released in the enzymic hydrolysis of a protein, which let us correlate the base consumption with the degree of hydrolysis, is a function of pH if we accept that the pK depends on the nature of the terminal amino acid and the nearest amino acids. If the distribution of the corresponding $pK_i$ is presumed to be normal, centred at $\mu$ with a standard deviation of $\sigma$, the relationship between the mean pK and pH was linear from the pH at which more than 95% of the $\alpha$-amino groups released were protonized to the pH at which less than 5% were protonized. The determination of this relationship for a given protein–protease system can be carried out by comparing the results of the titration of the original protein and the protein partially hydrolysed, as shown above in the hydrolysis of whey protein with Protex at 50 °C and pH = 8·0. The model developed in this work can easily be applied to any hydrolytic process involving the appearance of functional groups that are partially protonizable under the working conditions in order to follow the kinetics of the reaction via the consumption of the neutralizing agent required to keep pH constant.

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