Enzymatic Hydrolysis of Milk Proteins Under Alkaline and Acidic Conditions

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ABSTRACT

The pH-stat and osmometric methods were adopted for control of hydrolysis of casein and whey proteins by chymotrypsin, trypsin and pepsin. Experiments in alkaline medium showed that the mean pK values determined for amino groups in milk protein hydrolysates at 52°C were 7.11 for casein hydrolysates and 7.18 for whey protein hydrolysates. During hydrolysis in acidic medium the osmotic coefficients determined for casein and whey proteins enabled calculation of the calibration factor for the osmometer (1.05) which could be assumed as constant. Results will enable monitoring of DH during hydrolysis of milk proteins in such systems.

Key Words: dairy, milk proteins, enzymes, hydrolysis

INTRODUCTION

IT IS VERY IMPORTANT TO CONTROL THE reaction during production of enzymatic protein hydrolysates since stopping it at the right time determines the functional and sensory properties obtained (Antila, 1988; Vegarund and Langsrud, 1989; Ziajka et al., 1993). The extent of hydrolytic degradation of proteins is measured by the number of peptide bonds cleaved which, expressed in a percentage, is defined as the degree of hydrolysis or DH (Adler-Nissen, 1979; Novo Industri, 1980). Determination of DH by colorimetric methods is time-consuming. Therefore, routine rapid control of DH during hydrolysis is accomplished by two methods: pH-stat method or osmometry. To monitor extensive degradation of peptide chains, the pH-stat method is used for alkaline medium and osmometry for acidic medium (Novo Industri, 1980; Margot at al., 1994).

The principles of pH-stat method were developed in the Carlsberg Laboratory in Denmark (Jacobsen et al., 1957) and the method was further developed by Adler-Nissen (1979). DH can be calculated from the following equation, based on the base consumed during proteolysis (Novo Industri, (1980):

$$DH = B \cdot N_B \cdot (1/\alpha) \cdot (1/MP) \cdot (1/h_{tot}) \cdot 100$$

where: B=base consumption (mL); N_B=base normality; α=dissociation degree, $\alpha = 10^{pH - pK}/1 + 10^{pH - pK}$, MP=mass of protein substrate (g); h_{tot}=total number of peptide bonds (mM/g protein).

The principle of osmometry is based on the relationship between the number of peptide bonds released during hydrolysis and osmolality of the reaction mixture. The measurement of osmolality takes several seconds and the value read is converted to DH using the following formula (Adler-Nissen, 1986):

$$DH = [DC/(S\% \cdot f_{osm}) \cdot (1/\omega) \cdot (1/h_{tot}) \cdot 100$$

where: DC=osmolality (mOsm/kg); S%=substrate concentration; f_{osm}= conversion factor calculated on the basis of percentage content of dry matter in substrate (D%): $f_{osm}=1000/(100 - D\%)$; h_{tot}=totalnumber of peptide bonds (mM/g protein); ω=osmotic coefficient for peptides.

Our objective was to develop the pH-stat and osmometric methods for control of DH during hydrolysis of casein and whey proteins by chymotrypsin, trypsin and pepsin.

MATERIAL & METHODS

ACID CASEIN (93% PROTEIN IN DRY MATter) and whey protein concentrate WPC-80 (80% protein in dry matter) were used as protein substrates. Chymotrypsin (40 U/mg) and trypsin (44 U/mg) were from Sigma Chemical Co. (St. Louis, MO), and pepsin (15.5 U/g) was from Fluka Chemie AG (Buchs, Switzerland). Trinitrobenzenesulfonic acid (TNBS) and sodium dodecyl sulfate (SDS) were from Sigma Chemical Co. (St. Louis, MO).

Experiments in alkaline medium were carried out in a pH-stat (Metrohm, Basel, Switzerland) with automatic dosage of the base. The pH was adjusted using 4M and 0.1M NaOH.

The adjustment of pH during hydrolysis in acidic medium was made with 0.1M HCl. Cryoscopic osmometer (Trident, Warsaw, Poland) was used to measure osmolality.

pH-stat calibration

The calibration of pH-stat relied on determining the mean pK value for amino groups in milk protein hydrolysates. To determine the pH value at 52°C, two experiments were carried out for casein and whey protein hydrolysates using the pH-stat method at two pH values, pH_1 7.5 and pH_2 8.0. Protein substrates were hydrolysed by trypsin and chymotrypsin.

The amount of sodium hydroxide consumed during hydrolysis was converted to hydrolysis equivalents ($h_{pH\text{-}stat}$). Samples of hydrolysates were taken during proteolysis and were transferred into hot SDS, followed by determination of L-leucine equivalents (h_{TNBS}). After correlation of $h_{pH\text{-}stat}$ and h_{TNBS}, straight lines of slope *b* were obtained. The pK values were calculated from the following equation (Adler-Nissen, 1986):

$$pK = pH_2 + \log(b_1 - b_2) - \log(10^{pH2 - pH1} \cdot b_2 - b_1)$$

where: pH_1 and pH_2=pH values at which the hydrolysis was carried out ($pH_2 > pH_1$); b_1 and b_2=respective slopes of straight line.

Osmometer calibration

Osmometer calibration was carried out according to Adler-Nissen (1986) by correlating the hydrolysis equivalents as determined osmometrically (h_{osm}) with L-leucine equivalents, as determined with TNBS (h_{TNBS}). During the course of milk protein hydrolysis, samples were taken, one aliquot was directly transferred to hot SDS, followed by determining their content of a-amino groups with TNBS. The other aliquot was assayed for osmolality and h_{osm} was calculated from the equation (Adler-Nissen, 1986):

$$h_{osm} = [DC/(S\% \cdot f_{osm})] \cdot (1/\omega)$$

where: DC=osmolality, mOsm/kg; f_{osm}=conversion factor, calculated on a basis of the content of dry matter in the reaction mixture with substrate (D%); $f_{osm}=1000/(100 - D\%)$; S%=substrate con-

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centration (%), ω=osmotic coefficient of peptides.

Enzymatic hydrolysis of proteins

Casein and whey proteins were hydrolysed by chymotrypsinand trypsin at 52°C. The substrate concentration was 10% (v/v) and the enzyme to substrate ratio was 0.02. In the case of hydrolysis of milk proteins with pepsin, all parameters were the same except temperature (50°C). Conditions of hydrolysis were based on those of an earlier study (Dzwolak, 1995).

Determination of α-amino groups with TNBS method

Primary amino groups were determined in hydrolysate samples using TNBS with according to Adler-Nissen (1979).

Statistical analysis

Results (mean of four replicates) were submitted to calculation of correlation coefficients (*r*) and regression lines. The significance of the differences in data and the slopes of regression lines (*b*) were calculated by Student's *t* test.

RESULTS & DISCUSSION

Hydrolysis in alkaline medium

In all experiments a linear relationship was found between consumption of base and content of amino groups. The calibration of pH-stat was made together for trypsin and chymotrypsin hydrolysates. The results of calibration of pH-stat (Table 1, Fig. 1, 2) showed at pH 7.5, the slopes of straight lines were 2.47 (r=0.995) for casein hydrolysates and 2.05 (r=0.996) for those of whey proteins. The respective values at pH 8.0 were 1.99 (r=0.996) and 1.60 (r=0.997). The mean pK values for amino groups at 52°C were 7.11 for casein hydrolysates and 7.18 for whey protein hydrolysates.

The pK value of amino groups is not constant, changing depending on temperature of hydrolysis and size of peptides (Steinhardt and Beychok, 1964). A considerable effect of temperature on pK results from significant changes of entalphy of ionization of amino groups. Adler-Nissen (1986) reported that a change in temperature of hydrolysis by 10°C changed the pK value by about 0.23 pH units.

The pK values for di- and tripeptides were lower by about half than for polypeptides, being 7.5–7.8 on average at 25°C (Adler-Nissen, 1986). Thus at high degrees of hydrolysis (DH), *i.e.* at large amount of oligopeptides and free amino acids the pK value would differ from the pK value at low DH values (predominance of polypeptides). Adler-Nissen (1986) calculated the pK value on a basis of results obtained after hydrolysis of casein and soybean isolate by alkalase, which was 7.10 ±0.09 at 50°C.

Table 1—Calculations of pK values for amino groups in milk protein hydrolysates at 52°C

Substrate	pH	Equation	Correlation coefficient r	Slope b_1	Slope b_2	pK
Casein	7.5	y=2.47x + 0.15	0.995	2.47	—	—
	8.0	y=1.99x + 0.26	0.996	—	1.99	7.11
Whey proteins	7.5	y=2.05x + 0.22	0.996	2.05	—	—
	8.0	y=1.60x + 0.30	0.997	—	1.60	7.18
						mean 7.15

Our results confirmed the report by Richards (1956) that up to 17% DH the consumption of base in relation to the released amino groups levelled when ribonuclease was hydrolysed by Carlsberg subtilisin. As is known, endopeptidases hydrolize proteins to average DH values (<20%), therefore, the pK value could be assumed constant regarding this group of enzymes.

Hydrolysis in acid medium

Calibration of the osmometer was based on a determination of the calibration factor, which was the reciprocal of osmotic coefficient ω, which in turn equalled the slope of the calibration curve.

The results for osmometer calibration for casein and whey proteins, using pepsin were compared (Fig. 3, 4). Osmoticcoefficient ω changed with a change of solution concentration, which was determined by the nature of the solution. In electrolytes, non-electrolytes and ampholytes, ω deviated from unity, depending on concentration and type of solutes (Adler-Nissen, 1986). We assumed that the same situation occured in solutions of peptides and amino acids.

Chirife et al. (1980) reported that osmotic coefficient for α-amino acids, amides and glycylglycine was slightly lower than unity, the deviation being small. Adler-Nissen (1986) hydrolyzed soybean isolate by *Aspergillus niger* and *Aspergillus oryzae* proteases. The osmotic coefficient determined for peptides being cleaved during hydrolysis was 0.963 and calibration factor (1/ω) was 1.04.

In our study, the osmotic coefficients for casein and whey proteins were 0.950 and 0.948, respectively, and the calibration factor was 1.05. The straight lines (Fig. 3, 4) proved that the changes of ω depending on peptide concentration could be assumed insignificant and the calibration factor considered constant.

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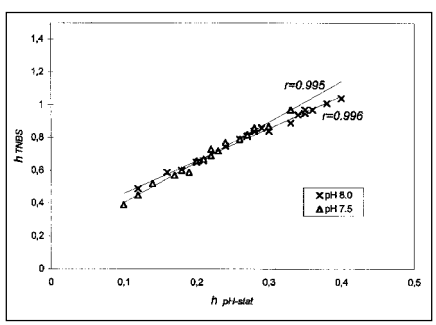

Fig.1–Calibration of pH-stat for trypsin and chymotrypsin hydrolysates of casein.

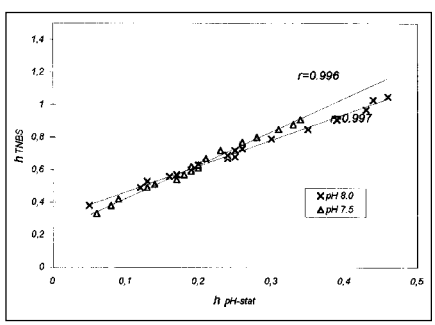

Fig.2–Calibration of pH-stat for trypsin and chymotrypsin hydrolysates of WPC.

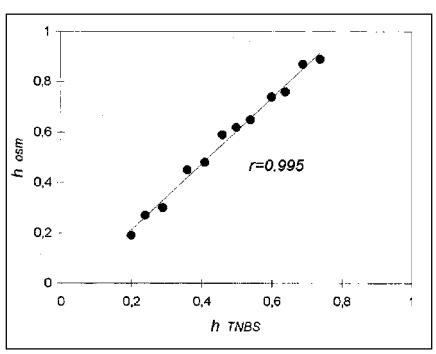

Fig.3–Calibration of osmometer for pepsin hydrolysates of casein.

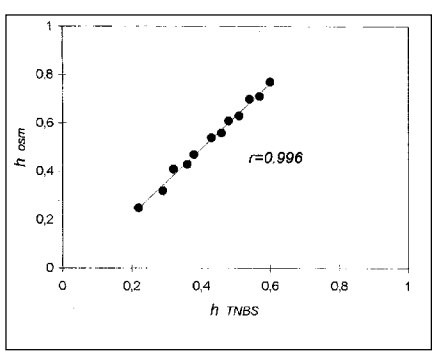

Fig.4 – Calibration of osmometer for pepsin hydrolysates of WPC.

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