ELSEVIER

PII: S0308-8146(97)00133-7

Food Chemistry, Vol. 61, No. 1/2, pp. 53–61, 1998

0308-8146/98 $19.00 + 0.00

On the kinetics of heat-induced deamidation and breakdown of caseinate

A. A. M. Metwalli[a] & M. A. J. S. van Boekel[b]*

[a]*Department of Dairying, Faculty of Agriculture, University of El-Minia, El Minia, Egypt*
[b]*Department of Food Science, Wageningen Agricultural University, PO Box 8129, 6700 EV Wageningen, The Netherlands*

(Received 13 March 1997; revised version received 16 June 1997; accepted 18 June 1997)

Caseinate (dissolved in a milk salt solution) was heated at temperatures between 110 and 145°C for 0–120 min. Five different concentrations of caseinate were studied ranging from 1–5% (w/w) solutions. The heated solutions were analysed for ammonia and for non-protein nitrogen content (NPN). The increase in ammonia concentration was taken as a measure for deamidation of the protein. It was established that the order of the reaction with respect to concentration (the 'true' order) was 1, as determined from the dependence of deamidation on initial concentration of caseinate. The order with respect to time could not be clearly established as the extent of deamidation was not higher than some 30%. However, the order with respect to time tended towards 2 rather than 1, which may be an indication for inhibition of deamidation as the reaction proceeds. The temperature dependence of deamidation was characterized from the Eyring equation; the activation enthalpy was found to be 92 kJ mol^{-1}, the activation entropy −70 J mol^{-1} K^{-1}. These results suggest a bimolecular reaction, in accordance with mechanisms of deamidation described in literature. The increase in NPN appeared to be much higher than that in ammonia. Ammonia (a part of the NPN fraction) amounted to only 10–15% of the NPN fraction. The NPN fraction increased considerably with heating intensity, for instance, to as high as 20% of total nitrogen (i.e. protein) after heating for 90 min at 140°C. This suggests considerable heat-induced protein breakdown resulting in small peptides and amino acids, or other low molecular weight nitrogen-containing breakdown products. The order with respect to concentration tended towards 1, while the order with respect to time tended again towards 2. The activation enthalpy for NPN formation was found to be 107 kJ mol^{-1} and the activation entropy −37 J mol^{-1} K^{-1}.

INTRODUCTION

Heating causes all kinds of chemical changes in proteins, such as peptide bond cleavage, dephosphorylation, racemization, oxidation, β-elimination, disulfide exchange, Maillard reactions in the presence of sugars, and last but not least deamidation of the amino acids glutamine (Gln) and asparagine (Asn) (Walstra and Jenness, 1984). Remarkably, however, not much is known about heat-induced deamidation of protein. In a study on degradation of urea to ammonia in heated milk (Metwalli *et al.*, 1996), it was noted that the contribution of ammonia formed due to milk protein deamidation was actually not known. It prompted us to study deamidation of casein in more detail. Deamidation of protein is of interest because it changes the properties of the protein, mainly because the electrostatic charge will change. Deamidation is considered to be one of the important mechanisms for thermo-inactivation of enzymes (Ahern and Klibanov, 1985; Daniel *et al.*, 1996). Another aspect of deamidation concerns the liberation of ammonia which could contribute to aroma formation via the Maillard reaction. This effect has been shown to be relevant, for instance, by Ho *et al.* (1994).

The limited data available in food science literature on heat-induced deamidation is on soy protein and egg white lysozyme (Zhang *et al.*, 1993*a*), soy protein (Zhang *et al.*, 1993*b*), egg lysozyme, casein and gliadin in a restricted water environment (Zhang *et al.*, 1993*c*). Wright (1991) gave a general review on deamidation of proteins but did neither include proteins relevant for foods nor heat-induced effects, but the review included quite a few results on peptide model studies. Current knowledge on mechanism and rate of deamidation has mainly been derived from studies on short model

*To whom correspondence should be addressed.

peptides (Manning *et al.*, 1989). Heat-induced deamidation of single amino acids has also been the subject of a study (Sohn and Ho, 1995).

General information about possible mechanisms of deamidation was given by Wright (1991). His main conclusions will be repeated here because they are useful to discuss our own results. Deamidation is an acid and base (nucleophile) catalysed hydrolytic reaction and requires a water molecule (much like ester hydrolysis): (see Fig. 1(A)). Consequently, Asn and Gln are maximally stable, as far as deamidation is concerned, near neutral pH, which is a crossover point for minimum deamidation rate at a pH that is suboptimum for both acid and base catalysis (Wright, 1991). However, if Asn and Gln are present in peptides and proteins, their rates of deamidation are increased as compared to the free amino acids. This is due to specific amino acid side chains that can act as catalysts: serine and threonine can act as general acid groups; aspartic acid, glutamic acid, histidine are nucleophiles at neutral pH, or function as general bases to activate nucleophiles; lysine and arginine can stabilize intermediates (Wright, 1991). Besides the general acid/base catalysed hydrolysis, the following mechanisms specific for protein/peptides can take place (Manning *et al.*, 1989; Wright, 1991):

1. Gln present at the amino terminus may deamidate to form pyrrolidone carboxylic acid with its own terminal amino group (this compound is present, for instance, in κ-casein).
2. Asn present at the carboxyl terminus may be subject to cyclization to form an anhydride.
3. At the sequences Asn-glycine, Asn-serine and Asn-alanine, a β-aspartyl shift mechanism is possible: (Fig. 1(B)). According to Wright (1991), this is a special case of nucleophilic attack on the Asn side chain group, the peptide bond of the succeeding residue (especially glycine) acting as the nucleophile. The resulting reaction intermediate is a succinimide ring, which easily breaks down to either the α- or β-isomeric aspartate products in the presence of water; the ratio of α- to β-isomer is 3:1. This mechanism does not occur at pH <4–5 where acid-catalyzed hydrolysis prevails, but it does at neutral and alkaline pH.
4. Peptide chain cleavage after an Asn residue via a mechanism similar to the β-aspartyl shift mechanism (Capasso *et al.*, 1996); this is not really deamidation but can lead to loss of Asn from the protein.

In view of the above mechanisms it is worth noting that deamidation of Asn occurs more readily than that

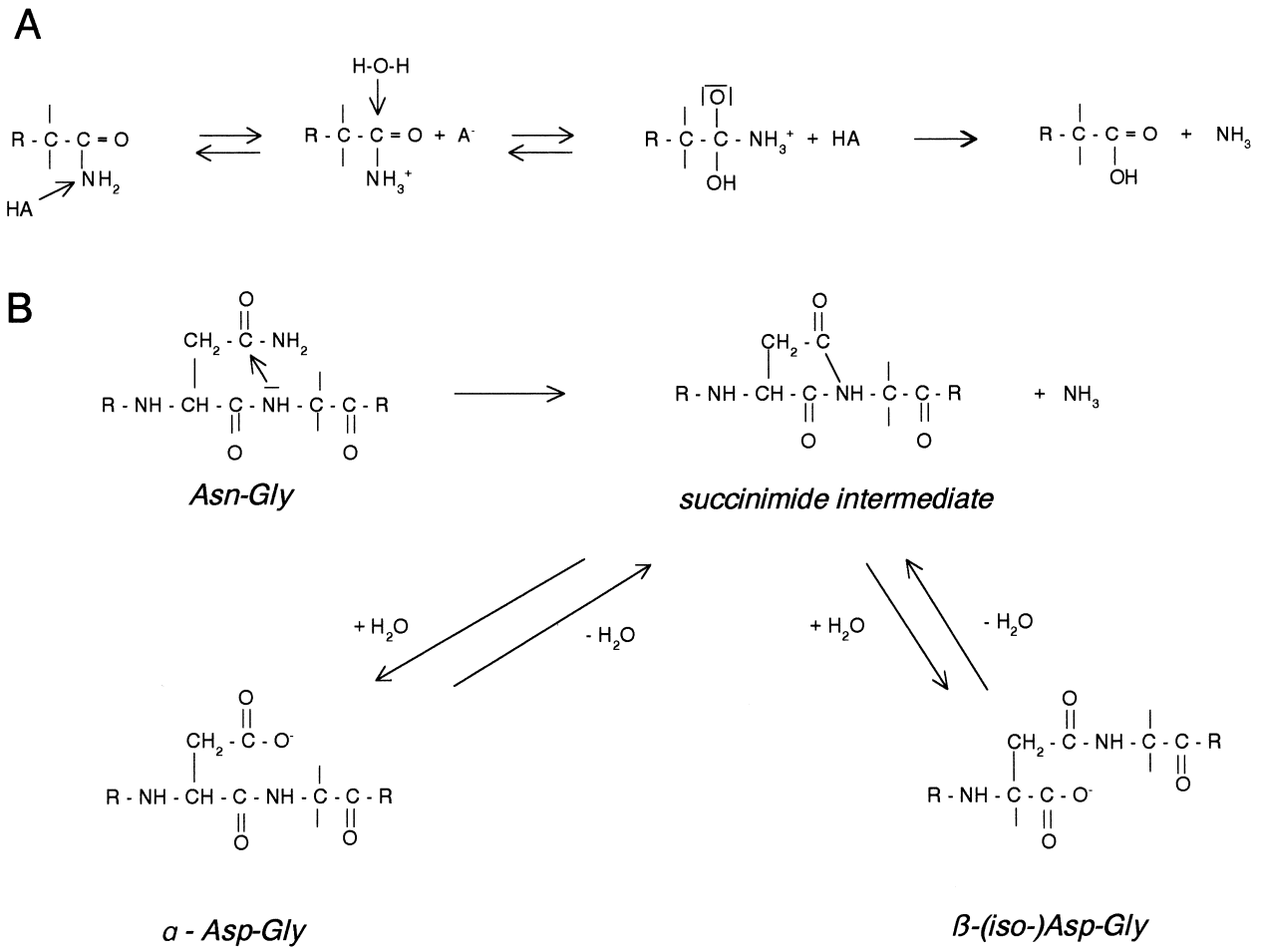

Fig. 1. Deamidation according to the hydrolysis mechanism (A) and according to the succinimide intermediate mechanism (B). HA represents a general acid, R represents a protein chain.

of Gln due to the fact that the amide group of Asn is closer to adjacent amino acid residues (Wright, 1991). Also Gln deamidation occurs via a (six-membered) ring intermediate glutarimide (Daniel *et al.*, 1996). In passing we note that, according to Sohn and Ho (1995), also free Asn deamidated more extensively than free Gln at 180°C. Apart from catalysis by neighbouring amino acids, catalysis can also occur by amino acids which are in the proximity of the Asn and Gln residues due to the specific three-dimensional structure of a protein.

It is clear from this short review that deamidation of proteins cannot be described as a simple hydrolysis process; it will depend on the type of amide, the specific amino acid sequence, the three dimensional structure of the protein. The pH affects protein structure as well as the mechanism of deamidation. The effect of pH is therefore difficult to predict beforehand. As far as casein is concerned, its three-dimensional structure is perhaps of less importance because casein has almost no secondary and tertiary structure. It tends however to aggregate, in milk in casein micelles (in which also colloidal calcium phosphate is involved), in caseinate solutions probably as aggregates of the size of casein submicelles. Due to this aggregation, some amino acid residues may be close to each other. The extent of aggregation is again pH dependent. Since the primary structure of the casein molecules is known (Walstra and Jenness, 1984), it is perhaps of interest to look at the occurrence of specific amino acid sequences in casein. The total amount of Asn in casein is 0.31 mol kg^{-1} and that of Gln 0.74 mol kg^{-1}, hence the total amount of amide is 1.05 mol kg^{-1} casein. (Zhang *et al.*, 1993*c*) found experimentally an amide content of 0.90 mol kg^{-1} casein for a sample with more than 90% protein, which is thus close to the theoretical value.) The four main components in casein are α_{s1}-, α_{s2}-, β- and κ-casein in the molar ratio of 4:1:4:1.6 (Walstra and Jenness, 1984). Table 1 gives an overview of the amide content of these four caseins. The specific sequences Asn-Gly, Asn-Ser and Asn-Ala do not occur frequently. Most of the serine residues are phosphorylated (SerP); it is not known whether phosphoserine has the same effect as serine on deamidation of a neighbouring Asn.

Table 1. Amide content of casein molecules

Casein	Asn content (mol kg^{-1})	Asn residues (mol^{-1})	Gln content (mol kg^{-1})	Gln residues (mol^{-1})	Specific sequences
α_{s1} (B variant)	0.34	7	0.74	13	Asn-Ser: 3 Asn-SerP: 2 Gln-Gly: 1
α_{s2} (A variant)	0.52	13	0.67	17	Asn-Ala: 2 Asn-Ser: 1 Gln-Gly: 1
β (A2 variant)	0.21	5	0.83	20	Asn-Ser: 1 Gln-Ala: 1 Gln-SerP: 1 Gln-Ser: 5
κ (B variant)	0.42	8	0.74	12	Not present

Zhang *et al.* (1993*a*,*b*) found for heat-induced deamidation of soy protein and lysozyme a slightly increased deamidation rate from pH 3 to 9 and a stronger increase in going from pH 9 to 11 (a complication being that soy protein is not well soluble near pH 4–5). The temperatures studied were 100, 115 and 130°C. Zhang *et al.* (1993*c*) found that deamidation rate increased with moisture content up to a certain level for soy protein, casein and lysozyme, but decreased for gliadin, the difference being of course that gliadin is not water soluble.

The objective of this research was to gain insight into the extent of protein deamidation as it may occur during sterilization of milk or milk-like products. It is known that heating induces an increase in the NPN (non-protein nitrogen) fraction of milk. Walstra and Jenness (1984) hypothesized that this increase could be due to deamidation because the amount of NPN released is nearly equivalent to the amide N of the protein. Consequently, we decided to study heat-induced changes in the NPN fraction as well. Because milk itself is too complicated a system (e.g. ammonia is formed not only because of deamidation but also due to urea decomposition), a simpler system of caseinate-buffer solutions was chosen because casein is the main protein in milk and its amide content is known, and changes in ammonia and NPN content can only derive from casein. The emphasis was on kinetics in the temperature range 110–145°C.

MATERIALS AND METHODS

Sodium caseinate was obtained from DMV, Veghel, NL. Caseinate was dissolved in concentrations 1–5% (w/w) in a synthetic milk salt buffer system (Jenness and Koops, 1962). Because sodium caseinate is obtained from large quantities of bulk milk, the sodium caseinate used had most likely the average composition of the variants α_{s1}-, α_{s2}-, β- and κ-casein in the molar ratio of 4:1:4:1.6 (Walstra and Jenness, 1984).

Samples (3.1 ml) were heated in stoppered stainless steel tubes (contents 3.3 ml) which were rotated in an oil bath at the required temperature. The heating-up time in this system was estimated to be 1 min. After a predetermined time, tubes were removed from the oil bath and immediately cooled in ice.

In several samples, the content of NPN and the concentration of ammonia was determined in the same sample, in other samples only the ammonia content was determined. NPN was determined by adding 15% trichloroacetic acid (TCA) such that the final concentration of TCA in the sample was 12%. The resulting protein precipitate was removed by filtration over red ribbon 589[5] filter paper. The nitrogen content of the filtrate was determined by the Kjeldahl method

(FIL-IDF, 1962) using semi-automatic equipment (Gerhardt Vapodest, DE). The NPN content was expressed as percentage of the total nitrogen present in the sample. The ammonia content was determined in the same filtrate by an enzymatic method (Boehringer, 1991). The pH was raised to 6–7 by adding 10 M KOH (results were not corrected for this small dilution effect).

Kinetic parameters were estimated by applying non-linear regression to the data (van Boekel, 1996).

RESULTS AND DISCUSSION

Caseinate solutions of various concentrations (Table 2) were subjected to heat treatments ranging from 110 to 145°C and heating times between 0 and 120 min. The coefficient of variation for estimation of ammonia contents was about 5% (including heating, sample treatment and subsequent analysis).

The data obtained were subjected to a kinetic analysis. The general rate law for chemical decomposition reactions is:

$$\text{rate} = -\frac{\mathrm{d}c}{\mathrm{d}t} = kc^n \qquad (1)$$

in which c is the concentration of the reactant (amide in this case), t reaction time, k the reaction rate constant and n the reaction order. Usually, this equation is integrated to give the time dependence of a reactant:

$$c_t = c_0[1 + (n_t - 1)kc_0^{(n_t-1)}t]^{\frac{1}{1-n_t}} \qquad (n_t \neq 1) \quad (2a)$$

$$c_t = c_0.\exp(-kt) \qquad (n_t = 1) \quad (2b)$$

in which c_t and c_0 are the concentration of reactant at time t and zero, respectively, and n_t the reaction order with respect to time. A typical example for the change in amide content is given in Fig. 2 as a function of heating time at 140°C. Because a straight line could not be fitted to the data, the order with respect to time (n_t) was apparently >0. It was, however, not possible to determine n_t exactly. The goodness of fit, as judged by the residual sum of squares, was slightly better for an order of 2 as compared to an order 1, but the differences were marginal (Fig. 2). The fact that the models of eqn (2) could be fitted to the data almost equally well for $0 < n_t < 3$ is probably due to the fact that the extent of deamidation was not very high. It is known that more than, say, 30–50% conversion is necessary before a differentiation in the value of n_t can be made (e.g. van Boekel and Walstra, 1995). The highest conversion in our study was around 30%. Zhang *et al.* (1993*b*) concluded that the deamidation reaction of soy protein was apparent first-order with respect to time (they reached conversion levels up to 50% at the highest pH and temperature), but they showed no other evidence than first-order plots; perhaps, a second-order equation would fit equally well to their data.

Table 2. Protein concentration (as determined from the nitrogen content ×6.38 after correction for NPN content) and amide content of the caseinate solutions (as calculated from the average composition of casein, Walstra and Jenness, 1984) used in the experiments

Solution	Protein content (g liter^{-1})	Asn content (mmol liter^{-1})	Gln content (mmol liter^{-1})	Amide content (mmol liter$^{-1)}$
1	9	2.79	6.66	9.45
2	18	5.58	13.32	18.9
3	27	8.37	19.98	28.35
4	34.7	10.63	25.37	36.0
5	45	13.95	33.3	47.25

It is interesting to note that the amount of ammonia released was never more than was present as amide N in Asn. This would be consistent with the idea that Asn is more easily deamidated than Gln (Wright, 1991). However, more research would be needed to confirm such a conclusion, for instance, by determining the amino acid composition of the protein after heating.

In order to get more information on kinetics, the order with respect to concentration (n_c) was determined from initial rates at the various concentrations studied; n_c is sometimes considered the 'true' order because information is confined to the beginning of the reaction without other possibly interfering reactions. (Since deamidation could be described reasonably well by a second-order eqn. (Fig. 2), initial rates were calculated by taking the derivative of the second-order equation at time zero, rather than drawing of tangents which was considered too subjective.) The common procedure for estimation of n_c is to take the logarithm of eqn (1): $\log(\text{rate}) = \log k + n_c \log c$ and apply linear regression; the slope is then n_c (Fig. 3). Taking logarithms may, however, lead to statistical difficulties (van Boekel, 1996), so we estimated n_c also by non-linear regression of eqn 1. The results are in Table 3. Although there are some differences in the outcome obtained via linear and non-linear regression the differences were not statistically significantly different. The results clearly point in the direction of $n_c = 1$. This is consistent with the hydrolysis mechanism, discussed above ((Fig. 1(A)), on the basis of which one would expect a pseudo first-order reaction, as the reactant water is present in large excess. In the case of the mechanism in which succinimide is formed as an intermediate, one would also expect a first-order reaction because formation of the succinimide intermediate is an intramolecular reaction, while hydrolysis of the intermediate (see Fig. 1) would be a pseudo-first order reaction because of the excess of water. To our knowledge, no other information about n_c is available in literature with regard to deamidation.

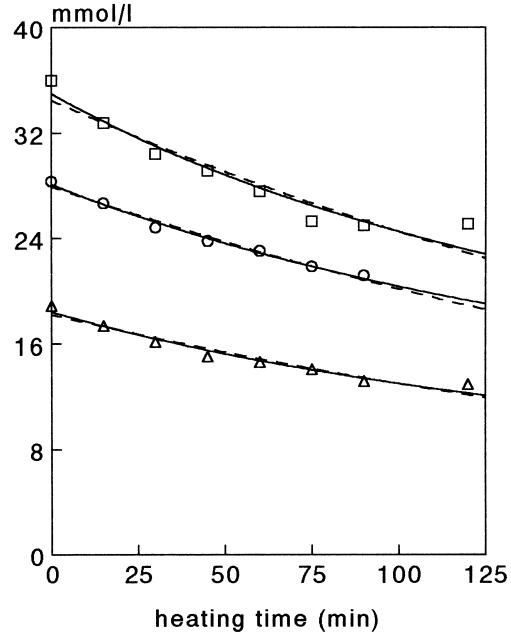

Fig. 2. Deamidation at 140°C at initial amide concentration 18.9 mmol liter^{-1} (△), 28.35 mmol liter^{-1} (○) and 36.0 mmol liter^{-1}(□). Closed line is for a first-order model, dotted line for a second-order model.

This result does not necessarily mean that n_t also equals 1 because the reaction may be autocatalytic ($n_t < n_c$) or inhibited ($n_t > n_c$) as the reaction proceeds. However, as noted above, our results are not conclusive about n_t, so we cannot really compare n_t with n_c. A catalytic or inhibiting effect cannot be excluded in this case, for

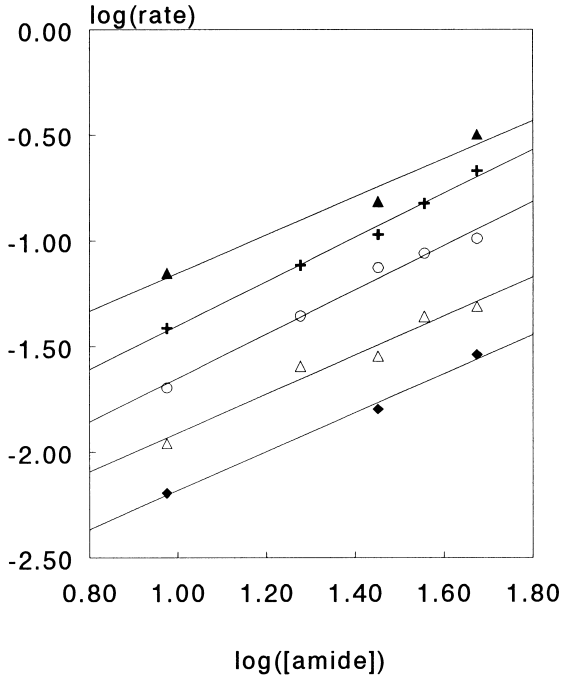

Fig. 3. Logarithm of initial rate of deamidation vs logarithm of amide concentration for 110(◆), 120(△), 130(○), 140(+) and 145°C(▲). The slopes of the lines indicate the order with respect to concentration (Table 3).

Table 3. Estimation of the order of the reaction with respect to concentration (n_c), ±(approximate) SE, the number of datum points per temperature studied is indicated between brackets

Temperature (°C)	Linear regression	Non-linear regression
110 (3)	0.93 ± 0.08	1.03 ± 0.12
120 (5)	0.92 ± 0.1	0.86 ± 0.14
130 (5)	1.04 ± 0.08	0.90 ± 0.11
140 (5)	1.04 ± 0.06	1.16 ± 0.09
145 (3)	0.90 ± 0.18	1.15 ± 0.29

instance, because there was a change in pH during heating (Fig. 4 gives an impression; pH measured at room temperature immediately after heating). The pH decrease is mainly due to heat-induced precipitation of calciumphosphate present in the milk salt solution (Walstra and Jenness, 1984); deamidation itself will increase the pH somewhat. Since deamidation is supposedly pH dependent the heat-induced pH decrease could have an effect. It should be realized that the pH was set and measured at room temperature, and the actual pH value during heating will be much lower (Walstra and Jenness, 1984). However, it is not possible to measure the pH above 100°C.

To get some idea about pH dependency, we did a few experiments at pH 7.5 (again set and measured at room temperature) for a casein solution containing 28.35 mmol liter^{-1} amide. The trend was that deamidation was somewhat stronger at pH 7.5 as compared to pH 6.5. Table 4 illustrates this with rate constants derived from a second-order model integrated with respect to time ($n_t = 2$ in eqn (2a)). However, the differences, if any, were not very large. Deamidation increases with pH according to literature (Zhang *et al.*, 1993*a*,*b*). Perhaps, the difference in pH in our experiments was not large enough to see a clear pH effect.

The temperature dependence of deamidation was estimated by applying the Eyring equation. It was decided to use the Eyring equation rather than the Arrhenius equation because the Eyring equation gives directly the activation entropy, which parameter may give a hint regarding the mechanism (van Boekel and Walstra, 1995). The Eyring equation is:

$$k = \frac{k_B T}{h} \exp\left(\frac{\Delta S^{\ddagger}}{R}\right) \exp\left(-\frac{\Delta H^{\ddagger}}{RT}\right) \qquad (3)$$

in which k_B is Boltzmann's constant, h Planck's constant, R the gas constant, T absolute temperature, $\Delta H^{\ddagger}$ activation enthalpy, $\Delta S^{\ddagger}$ activation entropy, respectively. The rate constants were obtained via eqn (1) taking $n_c = 1$ (cf. Fig. 3, Table 3), and the Eyring parameters were estimated using non-linear regression of eqn (3) to prevent statistical difficulties (van Boekel, 1996). There appeared to be no statistically significant differences between the various initial casein concentra-

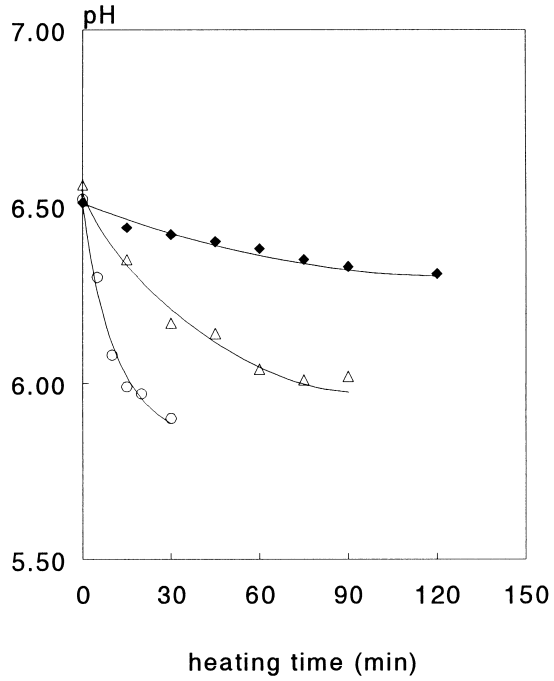

Fig. 4. Change in pH of caseinate solutions after heating at 110(◆), 130(△) and 145°C(○).

Table 4. Rate constants $\times 10^5 \pm$ approximate SD (obtained by non-linear regression of a second-order model) for deamidation of a caseinate solution with amide concentration 28.35 mmol liter^{-1} at pH 6.5 and 7.5

Temperature (°C)	pH 6.5	pH 7.5
110	2.0 ± 0.1	2.6 ± 0.1
120	3.6 ± 0.3	3.9 ± 0.3
130	9.4 ± 0.5	9.3 ± 0.5
140	13.5 ± 0.6	12.3 ± 1.0
145	19.2 ± 1.6	25.7 ± 1.5

tions and therefore all rate constants were used (Fig. 5). The results for the kinetic parameters thus obtained are $\Delta H^{\ddagger} = 92.0 \pm 13.6$ kJmol^{-1} (±95% confidence interval) and $\Delta S^{\ddagger} = -69.9 + 13.8$ J mol^{-1}K^{-1}(±95% confidence interval). The activation enthalpy found is quite normal for a chemical reaction, while the negative activation entropy is consistent with either of the two mechanisms depicted in Fig. 1, i.e. a bimolecular reaction. It is striking that the parameters found are almost exactly the same as those found for deamidation of a model hexapeptide (Patel and Borchardt, 1990; Geiger and Clarke, 1987). These authors demonstrated that the mechanism for deamidation was exclusively via the succinimide intermediate. Zhang *et al.* (1993*b*) determined activation energies from deamidation of soy protein at three temperatures (100, 115 and 130°C) at several pH values. They found 104 kJ mol^{-1} at pH 7; they did not report about activation entropy or the pre-exponential factor of the Arrhenius equation. Brennan *et al.* (1994), however, found an activation energy of 164 kJ mol^{-1} and a positive activation entropy of 146

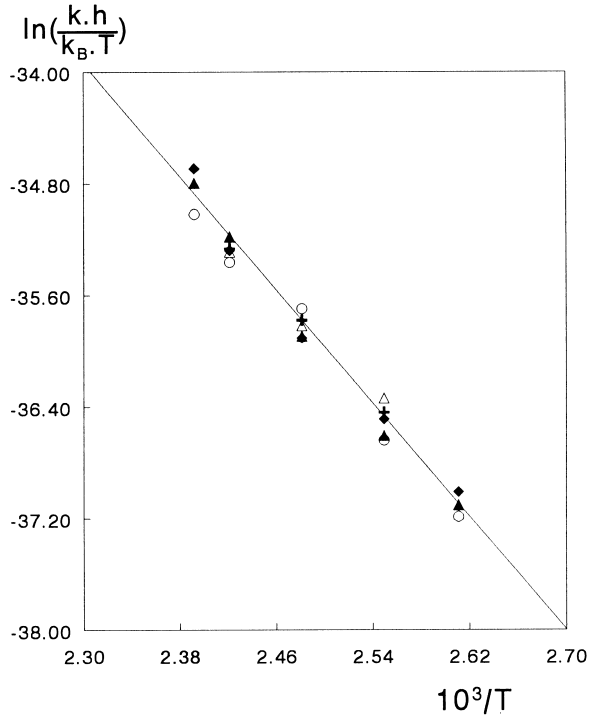

Fig. 5. Eyring plot depicting the temperature dependence of deamidation reactions of caseinate solutions with amide concentration 9.45(◆), 18.9(△), 28.35(○), 36.0(+) and 47.25(▲) mmol liter^{-1}.

J mol^{-1} K^{-1} for deamidation of a phosphocarrier protein. They concluded that these values indicated a local disruption of protein conformation around certain Asn residues during succinimide formation. Apparently, this local disruption was rate limiting, thus determining the kinetic parameters and giving rise to a positive activation entropy.

Besides ammonia, also the formation of non-protein nitrogen (NPN) was determined as a function of heating. The NPN fraction contains nitrogen compounds soluble in 12% TCA, among which ammonia. Other possible compounds are amino acids and small peptides, especially glycopeptides (Hindle and Weelock, 1970). Comparison of the formation of NPN with that of ammonia formed by deamidation may thus give an idea about heat-induced protein fragmentation besides deamidation. Peptide bond cleavage may even be linked to deamidation as Capasso *et al.* (1996) found that cleavage of a peptide bond next to an asparagine residue proceeds through similar pathways as deamidation, though at very different rates (deamidation rate being much higher). Our results indicated that the quantity of NPN components formed was much larger than the ammonia content (Fig. 6 gives an example). In general, the ammonia content was only 10–15% of the NPN content. NPN formation was studied for caseinate solutions between 110–145°C for several concentrations and at pH 6.5 and 7.5. Figure 7 gives an example for 3% caseinate solutions heated at pH 6.5. The proportion of NPN to total nitrogen (TN) was quite high, at

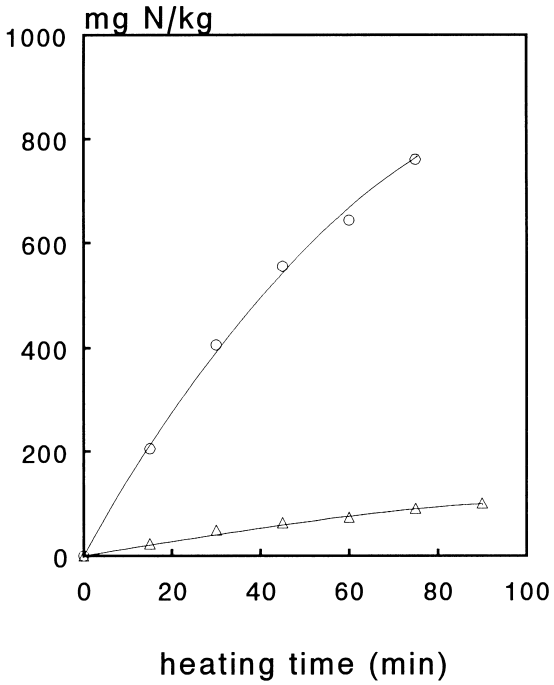

Fig. 6. Formation of NH_3(△) and 12% TCA-soluble nitrogen (NPN, O) as function of heating time at 140°C, pH 6.5.

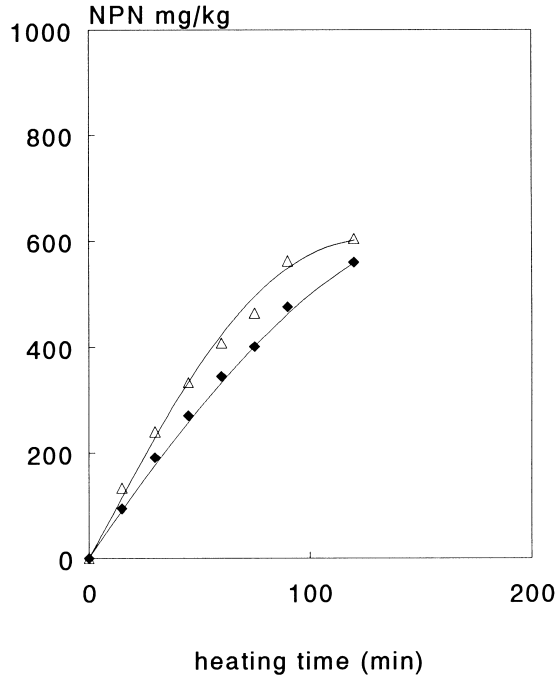

Fig. 8. Formation of NPN at 130°C at pH 6.5(◆) and 7.5(△).

140°C up to 20%. This means that a considerable portion of protein was degraded into small fragments; the nature of these fragments was not determined in this study. As with deamidation, NPN formation was slightly higher at pH 7.5 (Fig. 8). Also, a second-order equation ($n_t = 2$) fitted best to NPN formation but the differences were small for $0 < n_t < 3$. Saidi and Warthesen (1993) and Metwalli *et al.* (1996) studied NPN formation in heated milk and found zero-order kinetics. Belec and Jenness (1962) studied NPN formation for caseinate solutions and found also zero-order kinetics. Our results thus seem to deviate somewhat from literature. This is probably due, however, to the time–temperature combination we applied: deviation from zero-

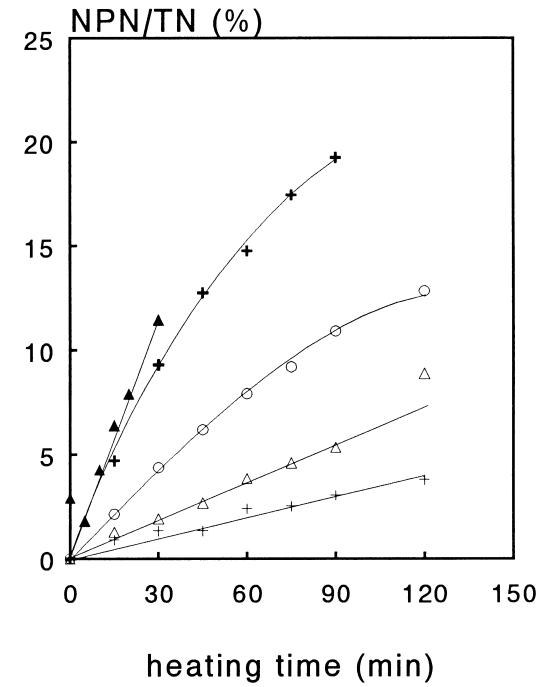

Fig. 7. Formation of NPN as fraction of TN as function of heating at 110(+), 120(△), 130(○), 140(+) and 145°C(▲).

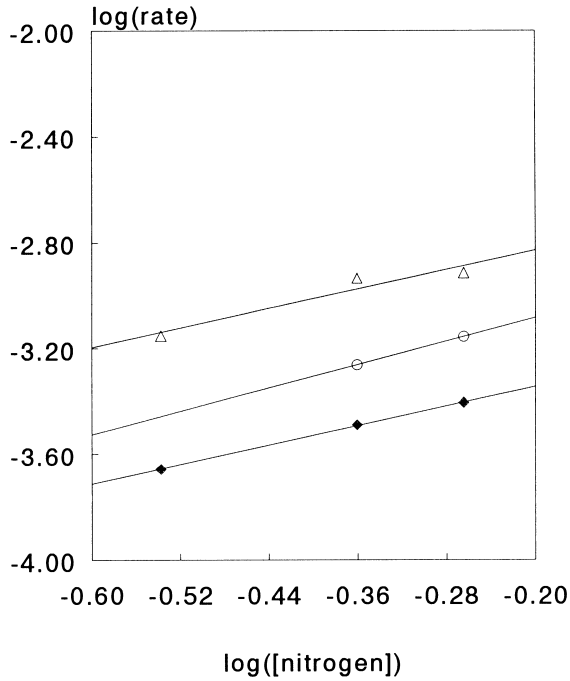

Fig. 9. Logarithm of initial rate of NPN formation vs logarithm of concentration of total nitrogen for 120(◆), 130(○) and 140°C (△). The slope for 120°C is 0.93, for 130°C 1.11, for 140°C 0.92.

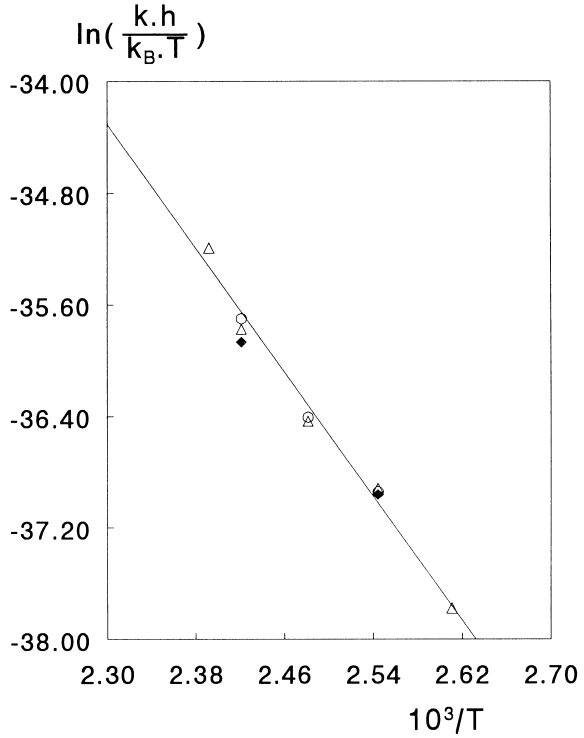

Fig. 10. Eyring plot for NPN formation of caseinate solutions heated at pH 6.5. Caseinate concentrations were 18(◆), 27(△) and 34.7 g liter^{-1}(□).

order kinetics becomes only obvious at the higher temperatures and longer heating times. The data from literature are mostly for lower temperatures and then a zero-order model fits equally well. The extent of NPN formation we found was in the same order of magnitude as reported in literature (Belec and Jenness, 1992; Saidi and Warthesen, 1993). The order with respect to concentration (n_c) tended to be 1 (Fig. 9), but the number of experiments was too small to draw definite conclusions. The activation enthalpy for NPN formation was 107.0 ± 27.6 kJ mol^{-1} ($\pm 95\%$ confidence interval) and the activation entropy was -37.4 ± 13.7 J mol K^{-1} ($\pm 95\%$ confidence interval), hence about the same as for deamidation (Fig. 10 shows the Eyring plot). The negative activation entropy points to a bimolecular reaction and may indicate that NPN formation is (mainly) due to hydrolysis of peptide bonds. Saidi and Warthesen (1993) reported a lower activation energy of 71 kJ mol^{-1} for NPN formation in heated milk; they did not mention a pre-exponential factor or activation entropy.

CONCLUSION

Heat-induced deamidation of casein occurs according to a (pseudo) first-order reaction, as found from the relation between initial rate and concentration. This is consistent with the reaction mechanism described in literature in which deamidation occurs via a succinimide intermediate. Also kinetic parameters describing temperature dependence point in this direction. However, the extent of deamidation was not large enough to establish the order with respect to time and any order between 0 and 3 would fit almost equally well. The extent of formation of non-protein nitrogen was much higher than ammonia formed due to deamidation, indicating that considerable protein fragmentation occurs because of heating.

ACKNOWLEDGEMENT

The authors would like to thank Mr H. Stempher for performing part of the experiments.

REFERENCES

Ahern, T. J. and Klibanov, A. M. (1985) The mechanism of irreversible enzyme inactivation at 100°C. *Science* **228**, 1280–1284.

Belec, J. and Jenness, R. (1962) Dephosphorization of caseins by heat treatment. I. in caseinate solutions. *Journal of Dairy Science* **45**, 12–19.

Boehringer Manual (1991) Test-kit for ammonia/urea determination. Boehringer Cat. No. 542.

van Boekel, M. A. J. S. and Walstra, P. (1995) Use of kinetics in studying heat-induced changes in foods. In *Heat-Induced Changes in Milk*, ed. P. F. Fox, pp. 22–50. International Dairy Federation, Brussels.

van Boekel, M. A. J. S. (1996) Statistical aspects of kinetic modelling for food science problems. *Journal of Food Science* **61**, 477–485,489.

Brennan, T. V., Anderson, J. W., Jia, Z., Waygood, E. B. and Clarke, S. (1994) Repair of spontaneously deamidated HPr phosphocarrier protein catalyzed by the l-iso-aspartate-(d-aspartate)-*O*-methyltransferase. *Journal of Biological Chemistry* **269**, 24586–24595.

Capasso, S., Mazzarella, L., Sorrentino, G., Balboni, G. and Kirby, A. J. (1996) Kinetics and mechanism of the cleavage of the peptide bond next to asparagine. *Peptides* **6**, 1075–1077.

Daniel, R. M., Dines, M. and Petach, H. H. (1996) The denaturation and degradation of stable enzymes at high temperatures. *Biochemical Journal* **317**, 1–11.

FIL-IDF standard 20. (1962) Determination of the total nitrogen content of milk by the Kjeldahl method. International Dairy Federation, Brussels.

Geiger, T. and Clarke, S. (1987) Deamidation, isomerization and racemization of asparaginyl and aspartyl residues in peptides. *Journal of Biological Chemistry* **262**, 785–794.

Hindle, E. J. and Weelock, J. V. (1970) The release of peptides and glycopeptides by the action of heat on cow's milk. *Journal of Dairy Research* **37**, 397–405.

Ho, C. H., Zhang, J., Hwang, H. I. and Riha, W. E. III. (1994) Release of ammonia from peptides and proteins and their effects on Maillard flavor generation. In *Maillard Reactions in Chemistry, Food and Health*, eds T. P. Labuza, G. A. Reineccius, V. M. Monnier, J. O'Brien and J. W Baynes. The Royal Society of Chemistry, Special Publication No. 151, pp. 126–130, Cambridge.

Jenness, R. and Koops, J. (1962) Preparation and properties of a salt solution which simulates milk ultrafiltrate. *Netherlands Milk Dairy Journal* **16**, 153–164.

Manning, M. C., Patel, K. and Borchardt, R. T. (1989) Stability of protein pharmaceuticals. *Pharmaceutical Research* **6**, 903–918.

Metwalli, A. A. M., Metwalli, N. H. and van Boekel, M. A. J. (1996) Effect of urea on heat-induced changes in milk. *Netherlands Milk Dairy Journal* **50**, 427–457.

Patel, K. and Borchardt, R. T. (1990) Chemical pathways of peptide degradation. II. kinetics of deamidation of an asparaginyl residue in a model hexapeptide. *Pharmaceutical Research* **7**, 703–711.

Saidi, B. and Warthesen, J. J. (1993) Heat and fermentation effects on total nonprotein nitrogen and urea in milk. *Journal of Food Science* **58**, 548–551.

Sohn, M. and Ho, Ch.-T. (1995) Ammonia generation during thermal degradation of amino acids. *Journal of Agricultural and Food Chemistry* **43**, 3001–3003.

Walstra, P. and Jenness, R. (1984) *Dairy Chemistry and Physics*. Wiley Interscience, New York.

Wright, H. T. (1991) Nonenzymatic deamidation of asparaginyl and glutaminyl residues in proteins. *Critical Reviews in Biochemistry and Molecular Biology* **26**, 1–52.

Zhang, J., Lee, T. C and Ho, Ch-T. (1993*a*) Comparative study on kinetics of nonenzymatic deamidation of soy protein and egg white lysozyme. *Journal of Agricultural and Food Chemistry* **41**, 2286–2290.

Zhang, J., Lee, T. C and Ho, Ch-T. (1993*b*) Kinetics and mechanism of nonenzymatic deamidation of soy protein. *Journal of Food Processing and Preservation* **17**, 259–268.

Zhang, J., Lee, T. C and Ho, Ch-T. (1993*c*) Thermal deamidation of proteins in a restricted water environment. *Journal of Agricultural and Food Chemistral* **41**, 1840–1843.