Cross-linking by transglutaminase changes neither the *in vitro* proteolysis nor the *in vivo* digestibility of caseinate

by N. Roos[1], P. Chr. Lorenzen[2], H. Sick[1], J. Schrezenmeir[1], E. Schlimme[2]

[1] Institute of Physiology and Biochemistry of Nutrition, and

[2] Institute for Dairy Chemistry and Technology, Federal Dairy Research Centre, P.O. Box 6069, D-24121 Kiel, Germany.

1. Introduction

The enzyme transglutaminase (EC 2.3.2.13) catalyses the linkage of peptide-bound glutamyl and lysyl residues among themselves and leads to high molecular protein aggregates. The techno-functional properties of food proteins can thereby be systematically changed, e.g. solubility, gelation properties, heat and emulsion stability. The variation of these properties through the cross-linking of proteins is being used increasingly in the meat, fish, milk and cereal-processing industries (1, 2, 3).

The ε-(γ-glutamyl) lysine bond which results from the cross-linking cannot be split by proteases in the gastrointestinal tract (4, 5, 6). Therefore, reduced protein digestibility and a reduction in the availability of the essential amino acid lysine is possible in transglutaminase-treated proteins. Admittedly, indirect nitrogen balance methods („protein efficiency ratio", „biological value") with rats (7) lead us to assume that the bioavailability of lysine from cross-linked casein is not reduced . However, up to now no studies have been conducted in order to determine the true digestibility of transgluatminase-treated proteins.

True protein digestibility can only be established when the dietary nitrogen can be differentiated from the endogenous nitrogen that is released into the lumen of the intestinal tract with secreta. This is made possible by the application of ^{15}N-labelled protein (8). The determination of protein digestibility results from the recovery of the stable isotope ^{15}N in the intestinal contents of the ileum after consumption of the protein to be examined.

Miniature pigs, which have a fistula at the end of the small intestine in order to collect ileal chyme, are particularly suitable for these types of digestibility studies, as the recovery of the administered protein marker can be measured in the ileal chyme. The values for the digestibility of milk proteins in humans correspond to those in miniature pigs (9, 10,11).

The aim of this study was, therefore, to determine the true prececal digestibility of transglutaminase-treated caseinate compared with that of native caseinate in miniature pigs.

2. Material and Methods

2.1 *In vitro* proteolysis

The production of caseinate and cross-linked caseinate was carried out as described by Lorenzen et al. (12). A sequential proteolysis of the isolated proteins was started first with pepsin for 120 minutes (substrate (S) = 5% (w/w), ratio of enzyme to substrate (E/S) = 1:100 (w/w), pH = 2.0, T = 37°C) and was subsequently continued with pancreatin (Sigma, Germany) for 120 minutes at pH 7.8. The identification of soluble amino nitrogen as a measure for proteolysis was carried out by Frister et al. (13).

2.2 ^{15}N-enriched caseinate

The production of ^{15}N-enriched milk is described in detail by Roos et al. (14). ^{15}N-labelled ammonium sulphate was continually infused into the rumen of a lactating cow for one week and the milk was collected. The casein was extracted from the labelled milk by acidic precipitation, and was then washed and lyophilised. The ^{15}N-labelling of the casein amounted to 0.4977 atom percent. The casein was dissolved in a highly dilute sodium hydroxide solution at a pH-value of 8.0 and half was cross-linked by transglutaminase (12). Both caseinate solutions were then lyophilised and ground.

2.3 Animals

The tests were carried out on four adult boars from the Göttingen Miniature pig stock of the Institute of Physiology and Biochemistry of Nutrition's experimental station Schaedtbek. These animals were fitted with a permanent ileum-T-cannula which enabled the collection of chyme at the end of the small intestine. The animals were 61-63 months old and weighed between 45 and 68 kg. During the test periods, they were kept in metabolic cages which enable a quantitative collection of chyme, faeces and urine. The room temperature was 19-21°C, the relative humidity 55-70%.

2.4 Diets

At least one week before the collection of chyme, the animals received a semi-synthetic standard diet (Table 1), which they were fed every morning (6 a.m.) and afternoon (3.30 p.m.), along with a litre of water. Between meals water was available ad libitum. The gross energy was 17.4 MJ per kg diet, corresponding to metabolisable energy of 15.3 MJ per kg diet. A metabolic maintenance requirement of 0.44 MJ per kg of metabolic body weight was taken to calculate daily amount of diet. In the evening before the start of the collection period, the animals received water only. On the following day at 6 a.m., a single meal of 200 g was given, which contained in each case only ^{15}N-enriched caseinate (diet 1, C) or ^{15}N-enriched caseinate, cross-linked by transglutaminase (diet 2, TgC) as protein component (see Table 1). Additionally, 10 g polyethylene glycol 4000 (PEG-4000, Merck, Darmstadt), dissolved in one litre of water, was added to the meal. PEG-4000 was used as an indigestible marker of the chyme's liquid phase. After the collection of chyme, the animals received the standard diet for a further five days, as faeces and urine was collected in this period.

2.5 Collection of samples

At intervals of 14 days, chyme was collected over 33 hours via the T-cannula of the distal ileum. After the insertion of a balloon catheter via the opening of the fistula, in the direction of the caecum, the balloon at the catheter end was filled with water and the digestive flow

in the caudal direction was thereby blocked. However, previous studies at the Institute have shown that this blockage is not total (15). Therefore, indigestible markers were added to the diet, as their recovery enabled the correction of the „chyme loss“ into the caecum. The intestinal contents, which were discharged intermittently via the fistula, were collected in a balloon which was attached to the T-cannula and – immediately after discharge from the fistula – were transferred into polyethelene bottles and frozen in liquid nitrogen. The time of collection and the quantity of chyme was registered. The chyme was stored at -20°C, was subsequently lyophilised, sieved (mesh width of the sieve: 0.5 mm) and pooled into eleven samples for every animal (0-2, 2-4, 4-6, 6-8, 8-10, 10-12, 12-15, 15-18, 18-21, 21-27, 27-33 hours postprandial). Before the collection periods, chyme samples without a blockage of the caecum were taken, in order to determine the natural abundance of ^{15}N in the ileal chyme.

Tab. 1: Compositon of the semi-synthetic diet

Components	Standard diet g/kg	Diet 1 (C) g/kg	Diet 2 (TgC) g/kg
Acidic casein[1]	150		
^{15}N-caseinate[2]		150	
^{15}N-caseinate, cross-linked[3]			150
Corn starch	290	290	290
Saccharose	240	240	240
Margarine	75	75	75
Lard	75	75	75
Cellulose	60	60	60
Mineral compounds and vitamins	80	80	80
Lactitol	30	30	30
Chromic oxid	4	4	4

[1] 0.368513 atom percent, natural ^{15}N-abundance

[2] 0.497689 atom percent, 13.71% nitrogen

[3] 0.497738 atom percent, 13.17% nitrogen

Urine und faeces samples were collected over 160 hours postprandially. Faeces samples were processed and stored in the same way as the chyme samples. After recording the total volume, aliquots of the urine samples were acidified with citric acid and stored at -20°C.

2.6 $^{15}N/^{14}N$ isotope ratio analysis

The ^{15}N-enrichment in diet, chyme, faeces and urine samples was determined by means of isotope ratio mass spectrometry (IRMS). For this purpose, samples which contained ca. 0.1 mg of nitrogen were burned at 950°C in the combustion unit of an elemental analyser in the presence of purified oxygen. The elemental analyser was connected to an isotope ratio mass spectrometer (delta E, Thermo Electron (Bremen) GmbH, Germany) via a trapping box interface. The NO_x from the burning process was

reduced over copper at 600°C (Dumas process) to N_2. The N_2 was trapped with molecular sieve (pore size 1 nm, Merck, Germany) in a trap at -196°C. After heating the trap to 70°C, the pressure of the N_2 in the trap was recorded and the gas was automatically transported into the inlet system of the mass spectrometer. The $^{15}N/^{14}N$ isotope ratio was measured eight times in reference to a standard laboratory gas, the values were converted to atom percent and related to the international standard (atmospheric nitrogen). With the help of acetanilide samples, with known nitrogen content, it was possible to determine the nitrogen content of the samples via the recorded pressure in the nitrogen trap. The whole process of burning the samples, isolating the nitrogen and measuring the isotope ratio was controlled by a computer (software: Control-It, Cordt-Computerdienste, Germany).

2.7 Chromic oxide determination

Chromic oxide was used as an indigestible marker for the solid phase of the chyme. The determination was carried out by Clarkson (16), whereby chromic oxide was oxidised to dichromate and was determined photometrically. The samples were solubilised with concentrated HNO_3 and 70% $HClO_4$ at 300°C, cooled down, filtered, and filled up to a known volume. The absorbance was measured at 440 nm and the content of chromic oxide was determined from a standard curve, which was prepared from standard solutions of dichromate.

2.8 Determination of polyethylene glycol(PEG) 4000

PEG-4000 is easily water-soluble and was used as an indigestible marker for the liquid phase of the chyme. PEG-4000 was determined by Hyden (17). After deproteinisation of the samples with barium hydroxide und zinc sulphate, the PEG-4000 was precipitated with trichloracetic acid and the turbidity was measured at 650 nm. The concentration of PEG-4000 in the samples was determined from a standard curve with standard solutions with known concentrations.

2.9 Calculations and statistics

The chyme flow was calculated from the wet weight of the intestinal contents which were emptied out via the ileum fistula per hour, corrected by the recovery of the indigestible marker PEG-4000.

$$\text{Chyme flow (g/h)} = \frac{w_C}{t * rec_{PEG}} * 100$$

w_C = wet weight of the chyme of one collection period (g)

t = duration of the collection period (h)

rec_{PEG} = recovery of the indigestible marker PEG-4000 (%)

The formula for the determination of the exogenous quantity of nitrogen in chyme (N_{EXO}) was deduced from the following steps:

(1) $N_C = N_{EXO} + N_{ENDO}$

$N_{ENDO} = N_C - N_{EXO}$

(2) $N_C * AP_C = N_{EXO} * AP_{EXO} + N_{ENDO} * AP_{ENDO}$

(1) in (2) $N_C * AP_C = N_{EXO} * AP_{EXO} + (N_C - N_{EXO}) * AP_{ENDO}$

$N_C * AP_C = N_{EXO} * AP_{EXO} + N_C * AP_{ENDO} - N_{EXO} * AP_{ENDO}$

$N_C * AP_C = N_{EXO} * (AP_{EXO} - AP_{ENDO}) + N_C * AP_{ENDO}$

$(N_C * AP_C) - (N_C * AP_{ENDO}) = N_{EXO} * (AP_{EXO} - AP_{ENDO})$

$$N_{EXO} = \frac{N_C * (AP_C - AP_{ENDO})}{AP_{EXO} - AP_{ENDO}}$$

N_{EXO} = amount of the exogenous (= dietary) nitrogen in chyme (in mmol)

N_{ENDO} = amount of the endogenous (= secreta) nitrogen in chyme (in mmol)

N_C = total nitrogen in chyme (in mmol)

AP_{EXO} = atom percent of the exogenous nitrogen

AP_{ENDO} = atom percent of the endogenous nitrogen in chyme (determined directly before collection)

AP_C = atom percent of the total nitrogen in chyme

From the constant administration of chromic oxide with the diet before and during the collection periods, a ‘steady-state’ of chromic oxide concentration in the chyme was aimed at, whereby a high recovery of the marker was to be achieved. From the concentration of nitrogen and chromic oxide in the diet, as well as their presence in the chyme of the 33 hour collection period, the prececal nitrogen recovery (rec_N) for 24 hours was determined:

$$rec_N (\%) = \frac{N_{EXO} * Cr_2O_{3_D}}{N_D * Cr_2O_{3_C}} * 100 * \frac{24}{33}$$

N_{EXO} = concentration of the exogenous nitrogen in chyme (in mmol/g dry matter)

N_D = concentration of nitrogen in the diet (in mmol/g dry matter)

Cr_2O_{3C} = concentration of the indigestible marker chromic oxide in chyme, (in mg/g dry matter)

Cr_2O_{3D} = concentration of the indigestible marker chromic oxide in the diet, (in mg/g dry matter)

By using PEG-4000 as an indigestible marker in order to calculate the nitrogen recovery, the absolute quantities of the PEG-4000 recovered during the collection period, and the exogenous quantity of nitrogen were applied:

$$rec_N\ (\%) = \frac{N_{EXO} * PEG_D}{N_D * PEG_C} * 100\ .$$

N_{EXO} = total exogenous nitrogen in chyme (in mmol)

N_D = quantity of nitrogen in the diet (i.e. in the single test meal, in mmol)

PEG_C = total quantity of PEG-4000 in chyme (in g)

PEG_D = amount of PEG-4000 in the diet (i.e. in the single test meal, in g)

The prececal nitrogen recovery (rec_N) is equated with the protein recovery. The prececal protein digestibility (pd_N) is then calculated from the difference between 100 and the prececal N-recovery (rec_N):

$$pd_N\ (\%) = 100 - rec_N.$$

The NPU (net protein utilisation) is the fraction of nitrogen of a protein which is retained in the body. It is calculated in the following way:

$$NPU\ (\%) = \frac{N_D - N_F - N_U}{N_D} * 100$$

N_D = amount of nitrogen in the diet (i.e. in the single test meal, in mmol)

N_F = amount of nitrogen in faeces (up to 160 h after feeding, in mmol)

N_U = amount of nitrogen in urine (up to 160 h after feeding, in mmol)

Results are expressed as arithmetic mean with standard errors. Differences between the two feeding groups were tested by means of ANOVA followed by Scheffé rank test. Differences were designated as significant with $p<0.05$.

3. Results and Discussion

The aim of the study was to determine the *in vitro* proteolysis of cross-linked caseinate as compared with caseinate not cross-linked, and also to determine their true prececal digestibility with the help of the stable isotope ^{15}N. Figure 1 demonstrates the sequential *in vitro* proteolysis of the caseinates with pepsin and pancreatin. The soluble amino nitrogen was used as a measure for the speed and extent of the proteolysis. The difference in the concentrations of soluble amino nitrogen before digestion corresponds to the fraction of amino groups blocked by the enzymatic cross-linking. The progress of the digestion of both substrates is the same. At the end of the digestion period with pepsin and pancreatin, the difference in the concentrations of soluble amino nitrogen in the proteolysates is as big as in the in the initial state of digestion. Lorenzen et al. (12) were able to show similar results for the digestion with trypsin. Thus, the cross-linking by transglutaminase does not hinder the *in vitro* digestibility of casein. The present study confirms the results of Chiba et al. (4), Yasumoto and Suzuki (5) and Seguro et al. (6, 7).

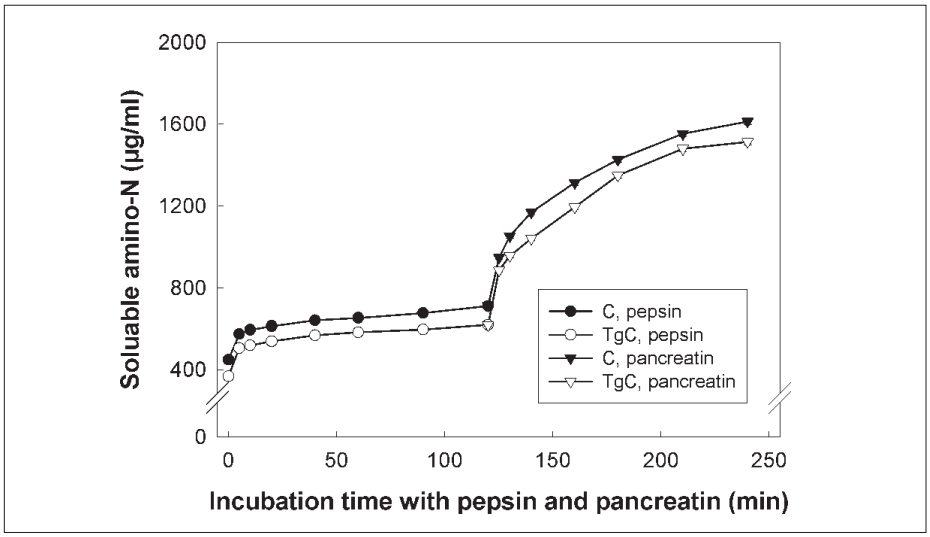

Fig. 1: *In vitro* proteolysis of caseinate (C) and transglutaminase-treated caseinate (TgC) by pepsin and pancreatin

The prececal digestibility is a measurement of the absorption of a food protein and is used as a criterion for the quality of the protein (18). To enable the measuring of the prececal digestibility, access to chyme from the ileum must be possible. Miniature pigs with an ileum-T-cannula have proved to be a suitable animal model for the determination of digestibility (10, 11, 19). During the collection periods, the digestive flow into the caecum is blocked. However, this blockage is not always complete which means that the flow of chyme must be corrected by the recovery of indigestible markers. Table 2 demonstrates the recovery of both indigestible markers in each animal dependent on the diet. The values differ considerably – both between animals and between feeding periods. This is easily explained by the fact that, during the sampling, the flow of chyme into the caecum was blocked to different degrees. However, this does not account for the low correlation between the markers in animal E804 with both kinds of test meal and in animal E805 with test meal C (Table 2).

Tab. 2: Recovery of the indigestible marker chromic oxide and polyethylene glycol 4000 (PEG-4000) at the end of the ileum after the feeding of caseinate or transglutaminase-treated caseinate to Göttingen Miniature pigs

Animal	Recovery			
	Chromic oxide		PEG-4000	
	C	TgC	C	TgC
	%			
E795	29.9	57.9	28.2	57.5
E796	77.0	70.8	90.3	74.5
E804	29.0	51.3	73.2	100.4
E805	44.4	128.7	80.4	110.7
mean ± SEM	45.1 ± 11.2	77.2 ± 17.6	68.0 ± 13.7	85.8 ± 12.1

In Figure 2 the flow rates for chromic oxide, PEG-4000 and exogenous nitrogen are shown. However, in this figure the exogenous nitrogen has not yet been corrected by the recovery of one of the two indigestible markers. Contrary to the expected steady state in the concentration of chromic oxide in the chyme through the constant feeding before collection periods, the concentrations of chromic oxide fluctuate strongly (Fig. 2A). PEG-4000 (Fig. 2B) was administered as a bolus with the test meal. This marker shows a high correlation with the progression of the exogenous nitrogen's flow rate (Fig. 2C) in both collection periods. Therefore, PEG-4000 recovery was used to calculate protein digestibility.

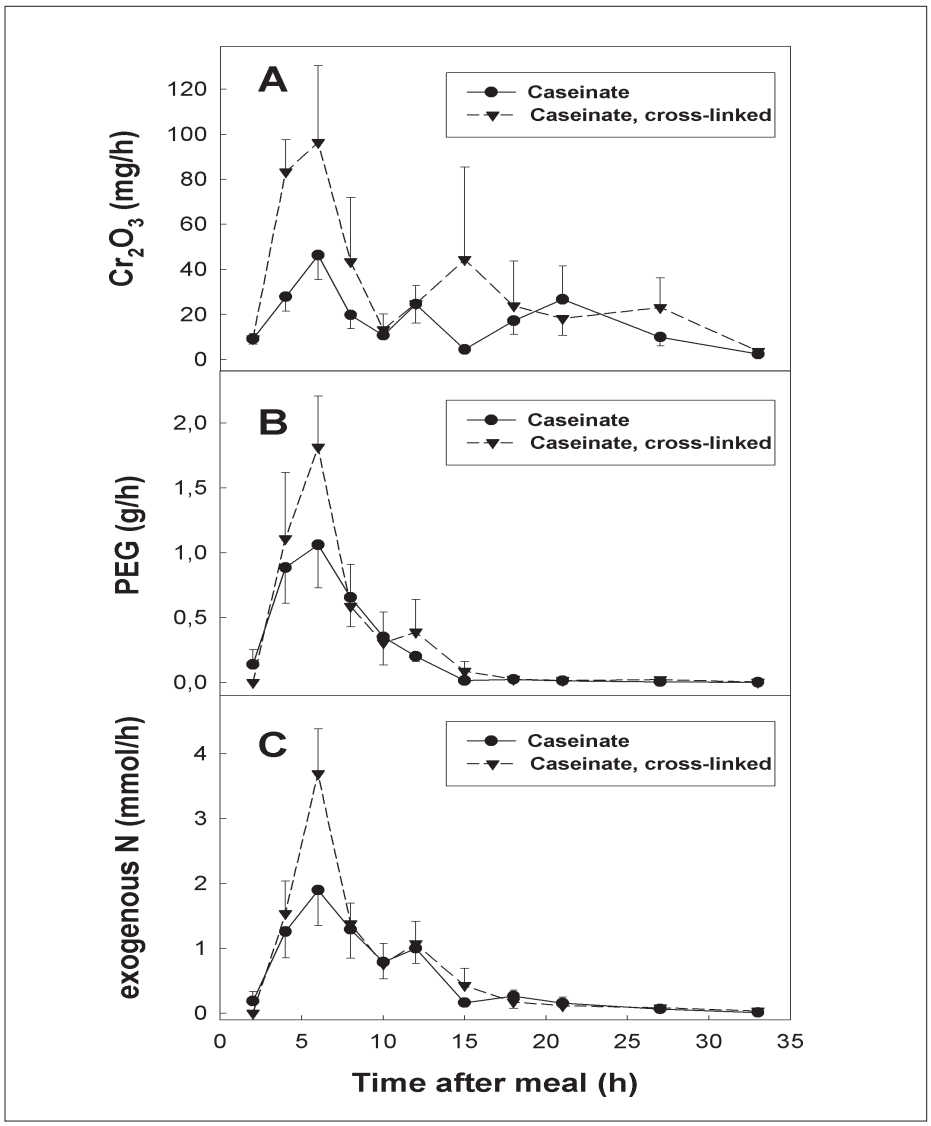

Fig. 2: Flow rates of chromic oxide (A), PEG-4000 (B) and exogenous nitrogen (C) at the end of the ileum after the feeding of caseinate or transglutaminase-treated caseinate to Göttingen Minature pigs

The cross-linking of caseinate by transglutaminase may not only lead to a change in the digestibility of food protein, but may also have in an influence on the digestive process through the changes in its functional characteristics, such as its ability to bind water or the gelation characteristic. The conformity in the times curves of the PEG-4000 flow rates in Figure 2B seems to suggest that the cross-linking of caseinate did not lead to delayed gastric emptying due to water binding and gelation. If this was the case, the maxima of the curves would have to displace each other. Admittedly, this effect would have to be very pronounced in order to make it visible at the end of the small intestine with the small resolution of the kinetic curve (2 hours in the front area of the curve). The conformity in the timing of the process also points out that the transit time for both diets is the same. The differing height of the maxima in Figures 2A, 2B and 2C can be explained by the fact that in animals, that received native caseinate as a test meal, accidentally higher chyme losses (i.e. less recovery of material) were registered in the caecum than in animals that received cross-linked caseinate.

Similarly, the cross-linking of casein by transglutaminase does not lead to an increase in the ileal chyme content. Figure 3 shows the flow rate of the chyme in the collection period, corrected by the recovery of the indigestible marker PEG-4000. The total chyme content in the caseinate group (C) was 623 ± 61 g, for the group that received cross-linked caseinate (TgC), 549 ± 35 g. The average quantity of dry substance in the chyme was not significantly different in either group (C: 8.9 ± 0.4 %; TgC: 9.3 ± 0.4 %) and gives no indication of diarrhoea or obstipation. The normal physiological process of digestion and absorption does not seem to be hindered by the feeding of cross-linked caseinate.

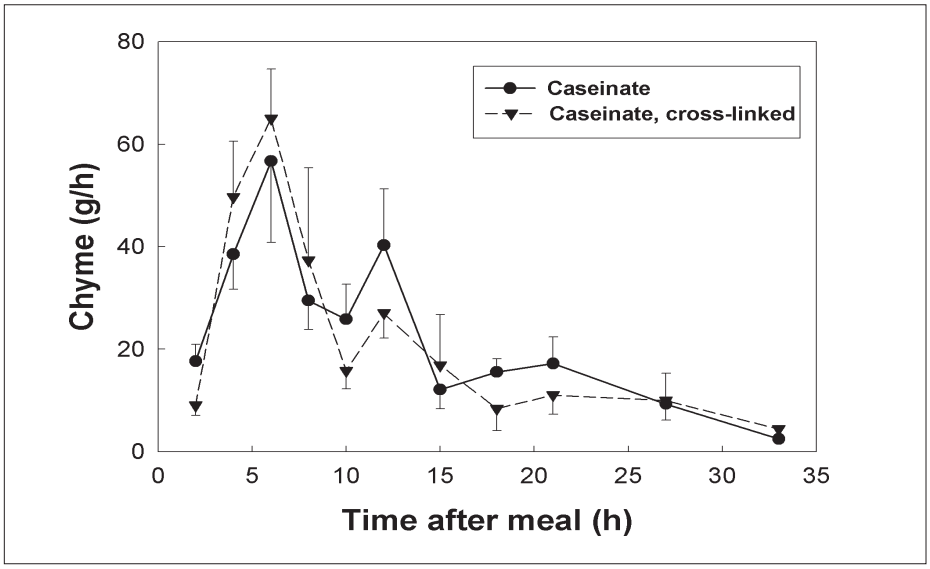

Fig. 3: Chyme flow at the end of the ileum after the feeding of caseinate or transglutaminase-treated caseinate to Göttingen Minature pigs, corrected by the recovery of the indigestible marker PEG-4000

The quantities of dietary nitrogen (exogenous nitrogen, Fig. 4A) and nitrogen from secreta (endogenous nitrogen, Fig. 4B) in the chyme did not show any significant difference between the two feeding groups. Thus, the feeding of transglutaminase-

treated caseinate does not seem to stimulate the release of endogenous secreta any differently than untreated casein. Stimulation has been observed in humans when proteins with a low digestibility were administered (20) and can lead to undesirable nitrogen losses. In Table 3 the values for nitrogen recovery for the entire collection time are summarised. From native caseinate (294 mmol of nitrogen in the test meal) 7.7 ± 0.6% (22.7 ± 1.8 mmol) of the nitrogen was recovered from the ileal chyme. This corresponds to a native caseinate digestibility of 92.3%. A digestibility of 91.9% results from the caseinate cross-linked by transglutaminase. The differences are not significant. The values correspond to casein digestibility values taken from literature which were also found using this method (14, 15).

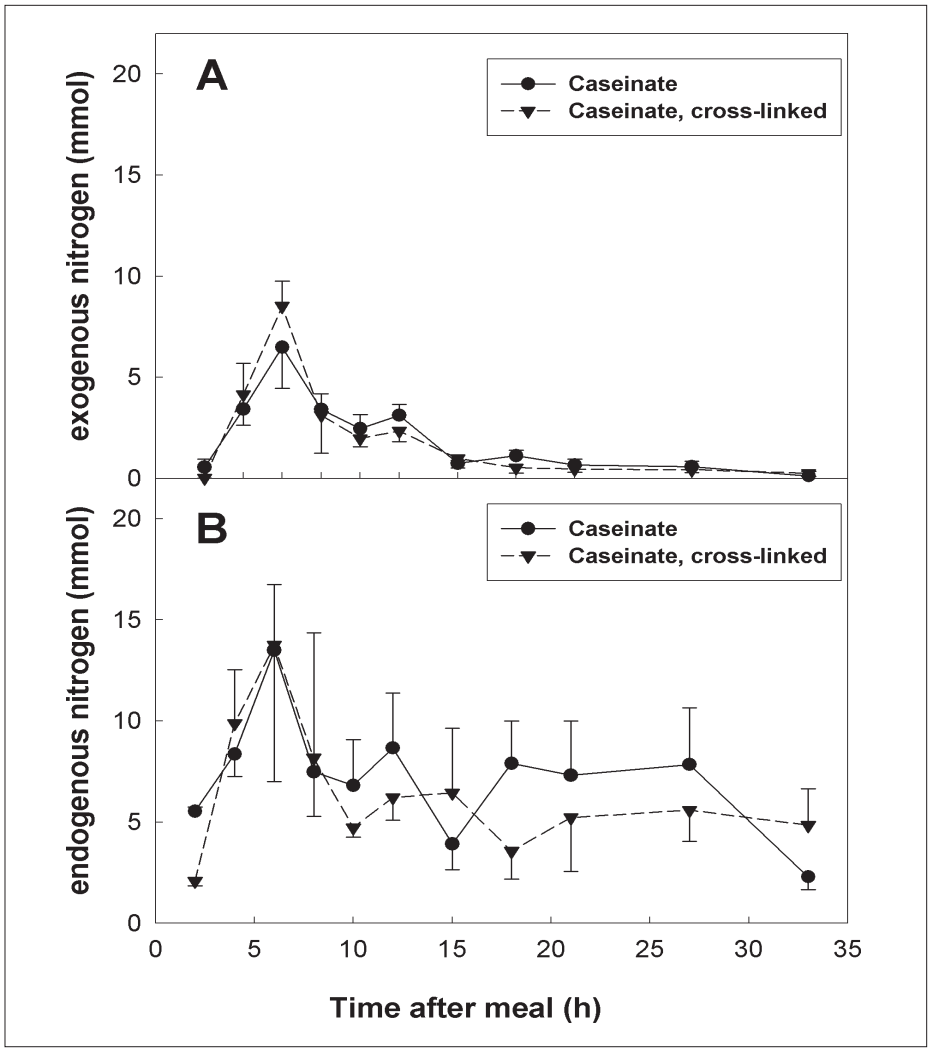

Fig. 4: Exogenous and endogenous nitrogen in the ileal chyme after the feeding of caseinate or transglutaminase-treated caseinate to Göttingen Minature pigs, corrected by the recovery of the indigestible marker PEG-4000

Tab. 3: Recovery of dietary nitrogen in the ileal chyme and the resulting protein digestibility after the feeding of caseinate or transglutaminase-treated caseinate to Göttingen Miniature pigs (mean ± SEM)

Protein	Dietary N	Exogenous N	Endogenous N	Recovery	Digestibility
		mmol		*%*	*%*
C	294	22.7 ± 1.8	79.7 ± 15.0	7.7 ± 0.6	92.3 ± 0.6
TgC	282	22.8 ± 1.8	69.5 ± 10.1	8.1 ± 0.6	91.9 ± 0.6

The cross-linking of the protein is made possible by the isopeptide bonds of the amino acids glutamine und lysine between neighbouring molecules. The proteases of the gastrointestinal tract cannot split the isopeptide bonds or can only do so to a limited extent (4, 5, 6, 21). However, experiments on rats have shown that ε-(γ-L-glutamyl)-L-[^{14}C]lysine can be absorbed in the small intestine and that the ^{14}C of the lysine was incorporated into plasma proteins (22). ε-(γ-glutamyl)-Lysine isopeptides are a substrate for the γ-glutamyl aminocyclotransferase (E.C. 2.3.2.4) of the kidneys (23). In this way the essential amino acid lysine would be available for protein metabolism. This explains studies such as that of Seguro et al. (7), which did not find limited lysine availability when transglutaminase-treated proteins were fed to rats. The data in Table 4 on the nitrogen retention of caseinate also seems to support these results. In the case of a lysine imbalance, caused by reduced availability of this amino acid, the retention of other amino acids would also be limited and this would manifest itself in a reduced NPU, i.e. a lower nitrogen retention in the body. However, the NPU of both proteins fed does not show any significant differences. This does not suggest reduced lysine availability in the feeding of transglutaminase-treated caseinate. Nevertheless, the determination of the NPU is only a very indirect method in order to evaluate lysine residues in the transglutaminase-treated caseinate. The direct proof of glutamyl-lysyl-isopeptides in urine would be a better demonstration of lysine losses.

Tab. 4: Content of nitrogen in diet, chyme, feaces and urine and resulting retention after the feeding of caseinate or transglutaminase-treated caseinate to Göttingen Miniature pigs (mean ± SEM)

Protein	Dietary N	Exogenous nitrogen			NPU
		in chyme	in faeces	in urine	
	mmol		*mmol*		*%*
C	294	22.7 ± 1.8	6.0 ± 1.2	72.2 ± 5.3	65.7 ± 2.4
TgC	282	22.8 ± 1.8	6.2 ± 0.6	68.1 ± 0.6	65.3 ± 6.8

To summarise, the results of this study show that the cross-linking of the main milk protein casein by transglutaminase inhibits neither its *in vitro* proteolysis, nor the digestibility in the intestinal tract of miniature pigs. Moreover, the change of the functional characteristic of caseinate does not seem to influence the physiology of the digestive process, e.g. by an increased transit time. The methods used could also not detect a reduced availability of lysine.

With regard to a nutritional evaluation of transglutaminase-treated food proteins we must refer to studies that establish a connection between the tissue transglutaminase of humans and celiac disease (24, 25, 26). Celiac disease is a disease of the intestine which occurs in genetically affected people through the consumption of gluten. It leads to partial damage of the absorbing mucosa and subsequently to nutrient malabsorption. Enhanced activity of the mucosal tissue transglutaminase and increased auto antibody levels against transglutaminase are characteristic for patients with celiac disease. It was shown that the transglutaminase deamidates proline-rich gliadine (component of the gluten). In particular, a proteolysis-resistant peptide with 33 amino acids significantly increased the reaction of gliadin-specific T-cells in the lamina propria (27). This ultimately leads to the damage of the mucosa. Whether cross reactivity can occur between the antibodies targeting the tissue transglutaminase and the transglutaminases used most often in food technology, has not yet been determined. Due to sequence homologies between human tissue transglutaminase and bacterial transglutaminases (BLAST, http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi), this cannot be ruled out at the present state of scienctific knowledge.

Acknowledgements

We thank the staff of the Animal Station of the Institute of Physiology and Biochemistry of Nutrition for sample collection and care of the animals and Ms. Kaschner from the Institute of Physiology and Biochemistry of Nutrition and Mrs. Splitzer from the Institute for Dairy Chemistry and Technology of the Federal Dairy Research Centre for expert technical assistance.

4. Literature

(1) Nielsen, P.M.: Reactions and potential industrial applications of transglutaminase. Review of literature and patents. Food Biotech. **9** 119-156 (1995)

(2) Kuraishi, C., Yamazaki, K., Susa, Y.: Transglutaminase: Its utilization in the food industry. Food Reviews Internat. **17** (2) 221-246 (2001)

(3) Armbrust, C., Werlein, H.-D., Watkinson, B.M.: Transglutaminase – Einsatz und Wirkung in Fischprodukten. Deutsche Lebensmittel-Rundschau **99** (5) 181-187 (2003)

(4) Chiba, H., Yoshikawa, M., Ikura, K., Sasaki, R.: Improvement of nutritional values and functional properties of food proteins by the transglutaminase action. Proceedings of the 6th International Congress of Food Science and Technology **2** 57-58 (1983)

(5) Yasumoto, K., Suzuki, F.: Aspartyl- and glutamyl-lysine crosslinks formation and their nutritional availability. J. Nutr. Sci. Vitaminol. (Tokyo) **36** Suppl. 1, S. 71-77 (1990)

(6) Seguro, K., Kumazawa, Y., Ohtsuka, T., Ide, H., Nio, N., Motoki, M., Kubota, K.: ε-(γ-Glutamyl)lysine: hydrolysis by γ-glutamyltransferase of different origins, when free or protein bound. J. Agri. Food Chem. **43** 1977-1981 (1995)

(7) Seguro, K., Kumazawa, Y., Kuraishi, C., Sakamoto, H. & Motoki, M.: ε-(γ-glutamyl)lysine moiety in crosslinked casein is an available source of lysine for rats. J. Nutr. **126** 2557-2562 (1996)

(8) Bergner H.: Determination of the protein quality of food and animal feed. Arch. Tierernähr. **45** (4) 293-332 (1994)

(9) Mahé, S., Huneau, J.F., Marteau, P., Thuillier, F. & Tomé, D.: Gastro-ileal nitrogen and electrolyte movements after bovine milk ingestion in humans. Am. J. Clin. Nutr. **56** 410-416 (1992)

(10) Gaudichon, C., Roos, N., Mahé, S., Sick, H., Bouley, C., Tomé , D.: Gastric emptying is a major regulatory system of nitrogen absorption kinetic from ^{15}N-labeled milk and yogurt in miniature pigs. J. Nutr. **124** 1970-1977 (1994)

(11) Gaudichon, C., Mahé, S., Roos, N., Benamouzig, R., Luengo, C., Huneau, J.F., Sick, H., Bouley, C., Rautureau, J. & Tomé, D.: Exogenous and endogenous nitrogen flow rates and level of protein hydrolysis in the human jejunum after [^{15}N]milk and yoghurt ingestion. Br. J. Nutr. **74** 251-260 (1995)

(12) Lorenzen, P.Chr., Schlimme, E., Roos, N.: Crosslinking of sodium caseinate by a microbial transglutaminase. Nahrung **42** (3/4) 151-154 (1998)

(13) Frister, H., Meisel, H., Schlimme, E.: Modifizierte OPA-Methode zur Charakterisierung von Proteolyse-Produkten. Milchwissenschaft **41** (8) 483-487 (1986)

(14) Roos, N., Pfeuffer, M., Hagemeister, H.: Labeling with 15N as compared with homoarginine suggests a lower prececal digestibility of casein. J. Nutr. **124** 12 2404-2409 (1994)

(15) Drescher, K.: Präzäkale Verdaulichkeit von ^{15}N- und Homoarginin-markierten Milchproteinen beim juvenilen und adulten Miniaturschwein. Inaugural-Dissertation, Fachbereich Veterinärmedizin, Justus-Liebig-Universität Gießen (1995)

(16) Clarkson, E.M.: A rapid method for the determination of chromium sesquioxide in faecal homogenates. Clin. Chim. Acta. **16** 186-188 (1967)

(17) Hyden, S.: A turbidimetric method for the determination of higher polyethylene glycols in biological materials. Ann. R. Agri. Coll. (Sweden) **21** 139-145 (1955)

(18) FAO/WHO: Report of the Joint FAO/WHO Expert Consultation on Protein Quality Evaluation, Food and Agriculture Organization of the United Nations, Rome, p. 26-34 (1990)

(19) Drescher, K., Roos, N., Pfeuffer, M., Seyfert, H.M., Schrezenmeir, J., Hagemeister, H.: Recovery of ^{15}N-Lactoferrin as compared to ^{15}N-casein is higher in the small intestine of suckling than of adult miniature pigs. J. Nutr. **129** 1026-1030 (1999)

(20) Mahé, S., Roos, N., Benamouzig, R., Davin, L, Gagnon, L., Gausserès, N., Rautureau, J., Tomé, D.: Gastrojejunal kinetics and digestion of [^{15}N]b-lactoglobulin and casein in humans: the influence of the nature and the quantity of the protein. Am. J. Clin. Nutr. **63** 546-552 (1996)

(21) Li Chan, E., Helbig, N., Holbek, E., Chau, S., Nakai, S.: Covalent attachment of lysine to wheat gluten for nutritional improvement. J. Agri. Food Chem. **27** 877-882 (1979)

(22) Raczynski G., Snochowski M., Buraczewski S.: Metabolism of ε-(γ-L-glutamyl)-L-lysine in the rat. Br. J. Nutr. **34** 291-296 (1975)

(23) Fink, M.L., Folk, J.E.: γ-Glutamylamine cyclotransferase. An enzyme involved in the catabolism of ε-(γ-glutamyl)lysine and other γ-glutamylamines. Mol. Cell Biochem. **38** 59-67 (1981)

(24) Dieterich, W., Ehnis, T., Bauer, M, Donner, P., Volta, U., Riecken, E.O., Schuppan, D.: Identification of tissue transglutaminase as the autoantigen of celiac disease. Nat. Med. **3** 797-801 (1997)

(25) van de Wal Y., Kooy Y., van Veelen P., Pena, S., Mearin L., Papadopoulos, G., Koning F.: Selective deamidation by tissue transglutaminase strongly enhances gliadin-specific T cell reactivity. J. Immunol. **161** 1585-1588 (1998)

(26) Molberg, O., Mcadam, S.N., Korner, R., Quarsten, H., Kristiansen, C., Madsen, L., Fugger, L., Scott, H., Noren, O., Roepstorff, P., Lundin, K.E., Sjostrom, H., Sollid, L.M.: Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. Nat. Med. **4** 713-717 (1998)

(27) Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., Gray, G.M., Sollid, L.M., Khosla, C.: Structural basis for gluten intolerance in celiac sprue. Science **297** 2275-2279 (2002)

5. Summary

Roos, N., Lorenzen, P.Chr., Sick, H., Schrezenmeir, J., Schlimme, E.: **Cross-linking by transglutaminase changes neither the *in vitro* proteolysis nor the *in vivo* digestibility of caseinate**. Kieler Milchwirtschaftliche Forschungsberichte **55** (4) 261-276 (2003)

18 Nutrition (transglutaminase, proteolysis, caseinate, digestibility)

The cross-linking of food proteins by transglutaminase changes technological characteristics in desired ways.

In order to examine whether cross-linking reduces protein digestibility, the *in vitro* proteolysis of transglutaminase-treated caseinate (TgC), compared with native caseinate (C), was measured. In addition, the prececal digestibility of these caseinates was studied in Goettingen Miniature pigs.

In vitro proteolysis was carried out with pepsin and pancreatin. The increase in soluble amino nitrogen as a measure for the extent of the digestion was similar for both substrates and gave no indication of a different proteolysis. For *in vivo* investigations four boars with a T-canula at the ileum were each given a semi-synthetic test meal containing 30 g of native or cross-linked caseinate, which were labelled with the stable isotope ^{15}N. The protein digestibillty was determined from the recovered ^{15}N in the ileal chyme. The indigestible markers chromic oxide and polyethylene glycol 4000 were added to the test meals in order to compensate for the chyme lost and to determine the flow-rate of the liquid phase of the digesta. Neither the quantity (C: 623 ± 61 g; TgC, 549 ± 35 g) nor the dry matter of the chyme (C: 8.9 ± 0.4%; TgC: 9.3 ± 0.4%) showed significant differences during the 33 hour collection period. Furthermore, the kinetics of the digesta-flow were similar. At the end of the small intestine 7.7 ± 0.6% and 8.1 ± 0,6% of the nitrogen of the administered native and cross-linked caseinate were recovered. This corresponds to protein digestibilities of 92.3% (C) and 91.9% (TgC), which are not significantly different. Likewise, the quantities of endogenous nitrogen, i.e. the nitrogen secreted into the gastrointestinal tract during digestion, were not significantly different after the two test meals.

The results of this study indicate that cross-linking of caseinate by transglutaminase changes neither the normal physiological process of digestion nor the protein digestibility.

Zusammenfassung

Roos, N., Lorenzen, P.Chr., Sick, H., Schrezenmeir, J., Schlimme, E.: **Die *in vitro* Proteolyse und *in vivo* Verdaulichkeit von Caseinat wird nicht durch Vernetzung mit Transglutaminase verändert**. Kieler Milchwirtschaftliche Forschungsberichte **55** (4) 261-276 (2003)

18 Ernährung (Transglutaminase, Proteolyse, Caseinat, Verdauung)

Die Vernetzung von Nahrungsproteinen mit Transglutaminase führt zu gewünschten Veränderungen ihrer technologischen Eigenschaften.

Um zu prüfen, ob die Vernetzung zu einer eingeschränkten Proteinverdaulichkeit im Gastrointestinaltrakt führt, wurde die *in vitro*-Proteolyse und am Modell des Göttinger Miniaturschweins die praecaecale Verdaulichkeit von Transglutaminase-behandeltem Caseinat (TgC) im Vergleich zu nativem Caseinat (C) untersucht.

Die *in vitro* Analysen wurden mit Pepsin und Pankreatin durchgeführt. Die Zunahme an löslichem Amino-Stickstoff war für beide Substrate ähnlich und gab keinen Hinweis auf eine unterschiedliche Proteolysierbarkeit. Für die *in vivo* Untersuchungen erhielten vier Eber mit Ileum-T-Kanüle in einer semisynthetischen Ration jeweils 30 g eines vernetzten oder nativen Caseinats, die mit dem stabilen Isotop ^{15}N angereicherten waren. Über die Wiederfindung des ^{15}N im Ileumchymus wurde die Proteinverdaulichkeit ermittelt. Die unverdaulichen Marker Chromoxid und Polyethylenglykol-4000 wurden den Rationen zur Korrektur von Chymusverlusten und zur Ermittlung der Flussrate der flüssigen Phase der Digesta zugefügt. Weder Menge (C: 623 ± 61 g; TgC, 549 ± 35 g) noch Trockenmasse des Chymus (C: 8,9 ± 0,4%; TgC: 9,3 ± 0,4%) zeigten signifikante Unterschiede während der 33-stündigen Sammlungsperiode. Auch die Kinetiken des Digestaflusses wiesen einen übereinstimmenden Verlauf auf. Am Ende des Dünndarms wurden im Falle des unbehandelten Caseins noch 7,7 ± 0,6% des verabreichten Stickstoffs wiedergefunden, nach Gabe des mit Transglutaminase vernetzten Caseinats 8,1 ± 0,6%. Dies entspricht einer Proteinverdaulichkeit von 92,3% (C) bzw. von 91,9% (TgC). Diese Werte weisen keinen signifikanten Unterschied auf. Die Mengen an endogenem, d. h. im Verlauf der Verdauung in den Darm sezernierten Stickstoff, waren ebenfalls nicht signifikant verschieden.

Die Ergebnisse dieser Studie weisen darauf hin, dass durch eine Vernetzung mit Transglutaminase der normale physiologische Ablauf der Verdauung und die Verdaulichkeit von Caseinat nicht verändert wird.

Résumé

Roos, N., Lorenzen, P.Chr., Sick, H., Schrezenmeir, J., Schlimme, E.: **La formation de liasons (rétification) avec la transglutaminase ne change ni la protéolyse *in vitro* ni la digestibilité *in vivo* du caséinate.** Kieler Milchwirtschaftliche Forschungsberichte **55** (4) 261-276 (2003)

18 Biochimie (transglutaminase, protéolyse, digestibilité)

La formation de liaisons entre des protéines alimentaires et la transglutaminase provoque les modifications désirées des propriétés technologiques.

Pour vérifier si la formation de liaisons mène à une digestibilité réduite des protéines dans le tract gastro-intestinal, la protéolyse *in vitro* et la digestibilité précécale de caséinate traité à la transglutaminase (TgC) ont été analysées sur le „porcelet miniature Göttingen“ par rapport au caséinate natif (C).

Les analyses *in vitro* ont été réalisées avec de la pepsine et de la pancréatine. L'augmentation en amino nitrogène était à peu près égale et n'a pas fourni d'informations sur une différence dans la capacité de protéolyse. Pour les examens *in vivo*, 30 g de caséinate réticulé ou de caséinate natif, enrichis par l'isotope stable ^{15}N, étaient administrés dans une ration semi-synthétique par une canule T- iléum à quatre verrats. La digestibilité protéique a été détectée moyennant ^{15}N recouvert dans le chyme de l'iléum. Les impulsions de marquage indigestibles chromoxide et polyéthylène glycol-4000 étaient ajoutées aux rations pour corriger les pertes de chyme et pour détecter le taux de flux. Ni les quantités (C: 623 ± 61 g; TgC, 549 ± 35 g), ni la matière sèche du chyme (C: 8,9 ± 0,4%; TgC: 9,3 ± 0,4%) indiquaient des différences signifiantes pendant la

période de collection de 33 heures. De même, les cinétiques du flux digestif révélaient un tracé concordant. Pour la caséine non-traitée, 7,7 ± 0,6% étaient recouverts à l'extrémité de l'intestin grêle après avoir administré du nitrogène, et 8,1 ± 0,6% après l'administration de caséinate traité à la transglutaminase. Cela correspond à une digestibilité protéique de 92,3% (C), respectivement de 91,9% (TgC). Ces valeurs ne révèlent pas de différences signifiantes. De même, les quantités de nitrogène endogène, c'est-à-dire le nitrogène sécrété dans l'intestin au cours de la digestion, ne révélaient pas de différence signifiante.

Les résultats de cette étude indiquent qu'une formation de liaisons avec la transglutaminase ne change rien au processus de digestion et à la digestibilité du caséinate.