Release of Protein-Bound *N*-ε-(γ-glutamyl)-Lysine during Simulated Gastrointestinal Digestion

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Abstract: *N*-ε-(γ-glutamyl)-lysine is a crosslinking amino acid formed in food mainly during treatment with microbial transglutaminase (mTG). The purpose of this study was to investigate to which amount isopeptides are detectable in a low-molecular weight peptide fraction after simulated gastrointestinal digestion. Casein, which had been enriched with N-ε-(γ-glutamyl)-lysine by mTG to different extents, was subjected to simulated gastrointestinal digestion and the resulting peptide mixture fractionated into a low- and a high molecular weight fraction (below or above 200–500 Da, respectively) using semipreparative gel permeation chromatography. N-ε-(γ-glutamyl)-lysine was analysed in these fractions by RP-HPLC after enzymatic hydrolysis and derivatisation with phenyl isothiocyanate. N-ε-(γ-glutamyl)-lysine was found nearly exclusively in the high-molecular weight fraction, indicating that dietary N-ε-(γ-glutamyl)-lysine present in mTG-modified food proteins is not available for absorption in the intestine.

Keywords: crosslinking; isopeptide; transglutaminase; simulated gastrointestinal digestion

INTRODUCTION

Treatment of proteins with microbial transglutaminase (mTG), a process applied in food industry to improve the technological properties of different foodstuffs (Jaros *et al.* 2006), leads to the formation of the crosslinking amino acid N-ε-(γ-glutamyl)-lysine (Glu_Lys). Thus, questions arise concerning the "metabolic fate" of such mTG-modified proteins. It is known that the digestibility of crosslinked proteins is impaired due to lowered accessibility by proteolytic enzymes (Savoie *et al.* 1991). The isopeptide bond can not be hydrolysed by pancreatic peptidases (Seguro *et al.* 1996). Different animal experiments, however, show that crosslinking of casein by mTG does not reduce protein digestibility (Seguro *et al.* 1996; Roos *et al.* 2004). Rats are able to use protein-bound and free Glu_Lys as a source of lysine (Seguro *et al.* 1996). The situation can be quite different in the human organism, since free Glu_Lys was shown to not be able to pass the intestinal barrier in an *in vitro* model (Brandsch *et al.* 2004). To gain further insight into the nutritional physiology of protein-bound Glu_Lys, mTG-modified casein was first digested *in vitro* and the digestion mixture fractionated into a high- (HMW) and a low-molecular weight (LMW) fraction. The content of Glu_Lys was analysed in both fractions in order to investigate whether Glu_Lys-containing and eventually absorbable peptides are formed.

MATERIALS AND METHODS

Casein was incubated in the presence of microbial transglutaminase (2.5 U/g casein) for 15, 45, and 120 min and oligomerisation was determined using gel-permeation chromatography (GPC) according to Lauber *et al.* (2000). Following mTG-treatment, casein samples were first digested with pepsin/mucin at pH 2.0 for 2 hours and then with trypsin/pancreatin/bile at pH 7.5 for 6 hours according to DIN 19738 (2004) in order to simulate gastrointestinal proteolysis. Samples withdrawn during the incubation were analysed by GPC using a column Superdex peptide HR 10/30 (Pharmacia, Freiburg, Germany) with phosphate-buffered sa-

line, pH 7.4 as the eluent. Flow rate was 0.5 ml/min, and the eluate was monitored at 280 nm.

For the semipreparative fractionation, 100 µl of the digested sample solution were subjected to semipreparative GPC using the conditions described above, but using water as the eluent. Three sample pairs each consisting of the combined fractions of four runs were prepared from the caseins. For the quantification of the amount of the isopeptide Glu_Lys, 2 to 3 mg of mTG-treated casein or one lyophilised sample from the fractionation experiments was hydrolysed enzymatically (Lauber *et al.* 2000). After hydrolysis, the samples were lyophilised and derivatised with phenyl isothiocyanate (PITC). Analysis was performed by HPLC using a phenyl-modified column and UV detection at 254/269 nm (Tarcsa & Fesus 1990).

RESULTS AND DISCUSSION

Incubation of casein with mTG for different times resulted in an increasing degree of oligomerisation as indicated in Table 1. The measured amounts of the isopeptide Glu_Lys were in the range of those naturally occurring in food (Seguro *et al.* 1996) or generated during mTG treatment (Lauber *et al.* 2000). The peptide patterns of the digested samples were monitored by GPC using a column suitable for short-chain peptide analysis. It should be emphasised that the digestion system utilised in this study does not comprise all of the possibilities of gastrointestinal digestion, since membrane-bound peptidases of the brush border are not included. The "digestibility index" was calculated from the peptide patterns measured at λ = 280 nm as the ratio of the peak area of peptides smaller than 1000 Da to the whole peak area. Calibration with short-chain peptides allowed us to calculate a theoretical elution time of 30 minutes for a peptide with a molecular mass of 1000 Da. The index was chosen based on the assumption that the shorter the peptides, the better they should be degradable by brush border peptidases and the better they could possibly be absorbed. Peptides exceeding three amino acids can not be transported actively by the intestinal peptide transporter (Brandsch *et al.* 2004). The digestibility indices of the crosslinked caseins decrease slightly with increasing degree of oligomerisation, but do not differ strongly. Thus, the digestibility of mTG-modified caseins is only slightly reduced despite the high degree of oligomerisation.

Semipreparative GPC turned out to be the method of choice for the reproducible isolation of a LMW fraction from the digested samples. Water was used as the eluent, as it could easily be removed from the isolates before subsequent analysis. Figure 1 shows chromatograms of a digested sample before and after separation by this system. The molecular weight cut-off of the fractionation system corresponds to 200–500 Da, which is the molecular weight range of di- and tripeptides. Absorption of peptides from such a fraction should be quite likely, even without the action of brush border peptidases.

We finally determined the contents of Glu_Lys and alanine in the HMW and the LMW fractions. Precolumn derivatisation with phenyl isothiocyanate was carried out on enzymatically hydrolysed samples (Tarcsa & Fesus 1990), and the phenyl thiocarbamoyl amino acids were analysed by RP-HPLC using a phenyl-modified HPLC column. No Glu_Lys was detected in the LMW fraction of native casein and the casein with the lowest degree of oligomerisation. With increasing Glu_Lys content, the percentage of isopeptide in the LMW fraction increased to a maximum liberation of 3.6% from the casein with the highest degree of oligomerisation. On the contrary, 21.0–26.0% of

Table 1. Analytical parameters, digestibility indices and amino acid distribution of the caseins investigated

Casein	Degree of oligomerisation (%)	Glu_Lys (mg/g)	Digestibility index (%)	Content in LMW fraction (% of total)	
				alanine	Glu_Lys
Native	16.8	0	90.0 ± 0.6	24.5	–
Cn15	39.4	1.2	89.4 ± 0.6	26.0	0
Cn45	51.7	2.5	89.4 ± 0.3	21.0	2.3
Cn120	71.5	3.3	88.1 ± 0.8	25.3	3.6

Values are means ± standard deviation

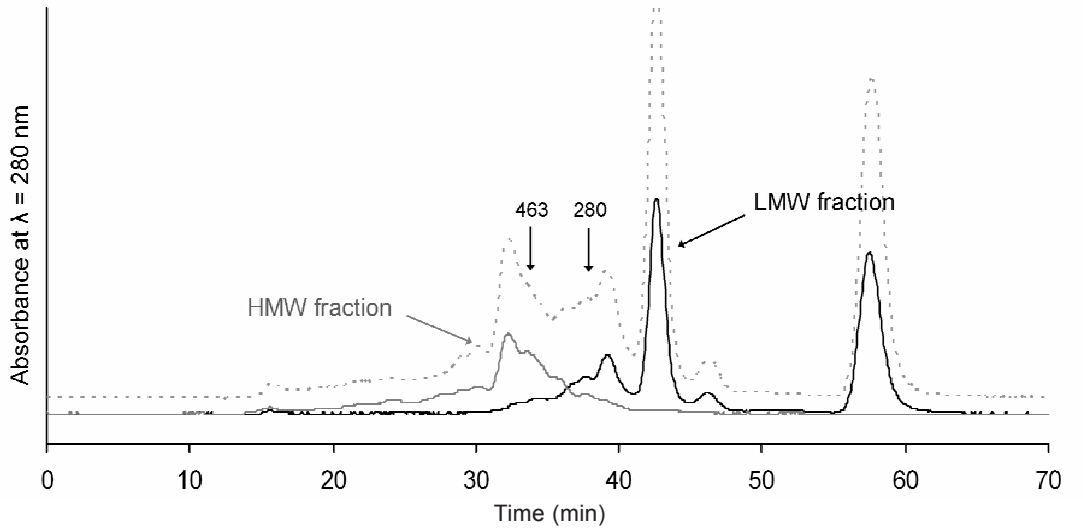

Figure 1. Gel-permeation chromatography of LMW and HMW peptide fractions obtained by semipreparative isolation compared to the original sample before fractionation. The elution times of two standard peptides (molecular weight 280 and 463 Da) are marked in the figure. Dotted line: original sample, grey line: HMW fraction, black line: LMW fraction

alanine could be found in the LMW fraction in each case.

The disproportionally low Glu_Lys content in the LMW fraction suggests that the liberation of Glu_Lys from proteins is strongly impaired during intestinal digestion. This should make it unavailable for intestinal absorption unless HMW peptides can be processed by brush border peptidases. Further studies are in progress in order to evaluate the absorption characteristics of peptide-bound Glu_Lys.

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