O-Methylisourea Can React with the α-Amino Group of Lysine: Implications for the Analysis of Reactive Lysine

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ABSTRACT: The specificity of O-methylisourea (OMIU) to bind to the ε-amino group of Lys, an important supposition for the OMIU-reactive Lys analysis of foods, feeds, ingredients, and digesta, was investigated. Crystalline L-Lys incubated under standard conditions with OMIU resulted in low homoarginine recoveries. The reaction of OMIU with the α-amino group of Lys was confirmed by MS analysis, with double derivatized Lys being identified. None of the changes in reaction conditions (OMIU pH, OMIU to Lys ratio, and reaction time) with crystalline L-Lys resulted in 100% recovery of homoarginine. The average free Lys content in ileal digesta of growing pigs and broilers was found to be 13% of total Lys, which could result in a significant underestimation of the reactive Lys content. The reaction of OMIU with α-amino groups may necessitate analysis of free Lys to accurately quantify reactive lysine in samples containing a large proportion of Lys with a free α-amino group.

KEYWORDS: guanidination reaction, reaction conditions, specificity, reactive lysine

INTRODUCTION

Protein-bound Lys with its free ε-amino group is considered the amino acid that is most susceptible to react with other compounds present in ingredients, foods, and feeds during thermal processing. One example is the reaction between amino groups and reducing sugars (Maillard reaction), resulting in the formation of Maillard reaction products. This reaction renders Lys unavailable for protein synthesis and concomitantly reduces the level of bioavailable Lys in foods and feeds. Analyzing Lys using conventional amino acid analysis provides an inaccurate estimate of bioavailable Lys, as early Maillard reaction products can revert back to Lys under the strong acidic conditions used to hydrolyze protein during amino acid analysis. A number of methods have been developed that can determine Lys possessing a free ε-amino group, i.e., reactive Lys, by reacting the latter group with a chemical reagent. In 1935, Greenstein reported that the chemical reagent O-methylisourea (OMIU) was specific for the ε-amino group of Lys in a guanidination reaction, which was corroborated in a number of subsequent studies. The guanidination reaction with OMIU results in the conversion of Lys to homoarginine, an acid stable amino acid which can be quantified using conventional amino acid analysis, thereby allowing the OMIU-reactive Lys content to be determined. The guanidination method for determining reactive Lys has been shown to accurately predict Lys availability in feed ingredients for growing pigs and has been extensively used to determine standardized ileal digestibility of reactive Lys for different foods and feeds such as wheat, soybean meal, heated skim milk powder, breakfast cereals, and cat foods. However, in 1967 Kimmel stated that the reaction of OMIU is specific for the ε-amino group if the α-amino group is blocked, suggesting that OMIU might be able to bind to the α-amino group of amino acids under certain conditions. Evidence for the nonspecificity of the guanidination reaction has been observed in the binding of OMIU to the free α-amino group of Gly and to a lesser extent of Met, Ser, Val, Leu, Phe, Glu, and Ala when OMIU is used to enhance MALDI mass spectra of peptides. In addition, the OMIU-reactive Lys content in diets containing crystalline L-Lys HCl was recently reported to be underestimated when analyzed using the guanidination reaction. The authors hypothesized that OMIU had reacted with the free α-amino group of crystalline L-Lys HCl under the specific conditions of the assay. Nonspecificity of OMIU for the ε-amino group of Lys may have implications when determining reactive Lys if foods, feeds, ingredients, and ileal digesta contain appreciable quantities of free and N-terminal Lys.

Since it has been hypothesized that OMIU also binds to the α-amino groups of amino acids in addition to the ε-amino group of Lys, the current study investigated the specificity of OMIU for the ε-amino group of crystalline L-Lys and the binding of OMIU to α-amino groups of selected crystalline amino acids. Reaction conditions (OMIU to Lys ratio, reaction time, and pH of the OMIU solution) for the specificity of OMIU to react with the ε-amino group of crystalline L-Lys were investigated. Practical implications of the results are assessed by

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examining the free Lys content of several food/feed ingredients and ileal digesta. The current study focused on ingredients used in feeds, but implications also account for food ingredients.

Materials and Methods

Materials and Terminology. Barium hydroxide octahydrate, crystalline L-Lys, L-Arg, L-Phe, L-Val, L-Ile, L-Thr, and Gly were obtained from Sigma-Aldrich (Castle Hill, Australia) and crystalline L-Lys HCl (78% L-Lys) from BDH Laboratory Supplies (Poole, England). The OMIU sulfate salt was obtained from Sigma-Aldrich (St. Louis, MO). All crystalline amino acids were reagent grade with a purity greater than 98%.

Free Lys was determined after extraction with 0.1 M HCl and precipitation of coextracted nitrogenous macromolecules by sulfosalicylic acid followed by centrifugation, separation and detection using ion-exchange chromatography employing postcolumn ninhydrin or o-phthalaldehyde derivatization.21 Total Lys was determined after acid hydrolysis in 6 M HCl for 24 h at 110 °C followed by separation and detection using ion-exchange chromatography employing postcolumn ninhydrin or o-phthalaldehyde derivatization.21 Reactive Lys was determined as being equivalent to the molar amount of homogargin quantified after incubation of the sample with OMIU followed by acid hydrolysis with 6 M HCl for 24 h at 110 °C.

Preparation of 0.6 M OMIU Solution. A 0.6 M OMIU solution was prepared according to the procedure described by Moughan and Rutherford22 except that 6 g of barium hydroxide octahydrate, instead of 4 g was added to approximately 16 mL of boiled distilled deionized water, which had been boiled for at least 10 min to remove carbon dioxide, in a centrifuge tube. Thereafter, 2 g of OMIU sulfate salt was added. The solution was cooled for 30 min at room temperature. The pH of the solution was adjusted to 10.6 with 0.1 M HCl and made up to 20 mL with boiled distilled deionized water.

Investigating the Binding of OMIU to Amino Groups Present in Crystalline Amino Acids. The binding of OMIU to amino groups of seven crystalline amino acids was investigated. Lysine, Arg, and Phe were selected because these amino acids have been reported to have the highest browning activity, i.e. most likely to react with sugars during processing.22 Valine, Ile, and Thr were selected because these amino acids are acid stable and frequently added to pig diets. Glycine was selected because it was previously reported to react with OMIU via its α-amino group.18,19 Unreacted and OMIU-incubated solutions of crystalline L-Lys HCl and other six selected crystalline amino acids (i.e., Arg, Phe, Val, Ile, Thr, and Gly) were analysed in duplicate to determine the amino acid content in non-OMIU incubated samples.

LC/MS Analysis of Guanidinated Crystalline Amino Acids. A 0.6 M OMIU solution was prepared as described above. Separate solutions of crystalline L-Lys and crystalline L-Tyr (0.0006 M) were incubated with OMIU for 3 days at room temperature in a shaker using an OMIU to amino acid ratio of either 10:1, 100:1 or 1000:1. Tyrosine was chosen as a model amino acid, in addition to Lys, because of its relatively high MW (181.19 g/mol) and low polarity, which both favor RP-LC detectability. Samples were analysed by an Acquity ultrahigh-performance liquid chromatography (UPLC) system (Waters, Milford, MA) using an Acquity UPLC BEH C18 column (2.1 × 150 mm, 1.7 μm particle size) with an Acquity BEH C18 Vanguard precolumn (2.1 × 50 mm, 1.7 μm particle size). Eluent A was 1% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid in Millipore water and eluent B was 100% acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Samples (1 μL) were injected into the column maintained at 40 °C. The analysis was conducted using the following elution profile: for OMIU incubated crystalline l-Lys, isocratic elution with 99.9% eluent A and 0.1% eluent B; for OMIU incubated crystalline L-Tyr, 0 to 2 min isocratic 99.9% eluent A, from 2 to 15 min linear gradient from 99.9% to 50% eluent A, from 15 to 20 min linear gradient from 50% eluent A to 99.9% eluent B, from 20 to 25 min isocratic at 99.9% eluent B, then re-equilibration to the initial conditions. The flow rate was set at 0.35 mL/min. The photodiode array detector was operated at a sampling rate of 40 points/s in the range 200–400 nm, resolution 1.2 nm. The SYNAPT G2Si mass spectrometer was operated in positive ion mode, capillary voltage 3 kV, sampling cone 30 V, source temperature 150 °C, desolvation temperature 500 °C, cone gas flow (N2) 200 L/h, desolvation gas flow (N2) 800 L/h, acquisition in the Full Scan mode, scan time 0.3 s, acquisition range 150–2000 m/z. The MS was calibrated using NaI (m/z range: 100–2000). The MS data were processed using the software MassLynx v 4.1 (Waters, Milford, MA).

Influence of Reaction Time and OMIU to Lys Ratio on Guanidination of Crystalline l-Lys. The influence of OMIU to Lys ratio and reaction time on the guanidination of crystalline L-Lys was assessed using a 4 × 3 factorial arrangement with four OMIU to Lys ratios and three reaction times. The OMIU to Lys ratios were 1:5:1 (optimal to convert crystalline L-Lys to homogargin12), 10:1 (reported to be optimal for casein24), 100:1, and 1000:1.13 The reaction times were 1, 3, and 7 days with the remaining reaction conditions as described above.

Influence of OMIU to Lys Ratio and OMIU pH on Guanidination of Crystalline l-Lys. The influence of pH of the OMIU solution and OMIU to Lys ratio on guanidination of crystalline L-Lys was assessed using a 7 × 2 factorial arrangement with seven pH levels and two OMIU to Lys ratios. The pH values ranged from 8.6 to 11.0 with 0.4 increments, with pH 9.0 and 10.6 being the pKc values for the α- and ε-amino groups of Lys, respectively. The OMIU to Lys ratios were 10:1 and 1000:1. A reaction time of 3 days was used with the remaining reaction conditions as described above.

Analysis of Crystalline L-Lys HCl and a Mixture of Crystalline Amino Acids Using Two OMIU to Amino Acid Ratios during Guanidination. L-Lysine HCl (78% l-Lys), i.e. a form of crystalline l-Lys that is supplemented to diets, and an equimolar mixture of the other six selected crystalline amino acids (i.e., Arg, Phe, Val, Ile, Thr, and Gly) were analysed using an OMIU to amino acid ratio of 10:1 and 1000:1, an OMIU pH of 10.6 and a reaction time of 3 days. Unreacted and OMIU-incubated solutions of crystalline L-Lys HCl and the mixture of six crystalline amino acids were analyzed in duplicate using the HPLC system as described above.

Examining the Free Lys Content in Selected Protein Sources. Data on the free Lys as percentage of total Lys for 44 different food/feed ingredients were obtained from Ajinomoto Eurolysine s.a.s.25 The free and total Lys contents were determined by Ajinomoto Eurolysine s.a.s. using the procedures described above.

Free Lys Content in Ileal Digesta Collected from Pigs and Broilers Fed Protein-free or Selected Protein-Containing Diets. Samples of ileal digesta were selected based on the protein source present in the experimental diets and the method used to collect the digesta during animal trials with growing pigs or broilers previously conducted at the Riddet Institute (Palmerston North, New Zealand) and Animal Nutrition group of Wageningen University (Wageningen, The Netherlands).

With regard to the growing pig trials, samples were obtained from five experiments. In the first experiment, diets contained soybean meal or rapeseed meal as the sole protein source and were each fed to seven (steered ileo-cecal valve) cannulated growing pigs (n = 14). Crystalline L-Lys HCl was added to the rapeseed meal diet. In the second experiment (H. Chen, Wageningen UR Livestock Research, personal communication), a protein-free diet was fed consisting of corn starch, dextrose, arabecel (fiber source from J. Rettenmaier & Sohne Group, Rosenberg, Germany), soy oil and vitamins/minerals.
marker. In the same study, soybean meal or rapeseed meal was added as the sole protein source to the experimental diets at the expense of corn starch. Each diet was fed to three growing pigs and ileal digesta was collected at slaughter (n = 9). In the third experiment (S. M. Rutherfurd, Riddet Institute, personal communication), a protein-free diet (corn starch, sugar, cellulose, soybean oil, vitamins/minerals/ marker) was fed to growing pigs and ileal digesta collected at slaughter. The ileal digesta of four pigs was pooled based on freeze-dry matter content (n = 1). In the fourth experiment (S. M. Rutherfurd, Riddet Institute, personal communication), a protein-free diet (wheat starch, sucrose, cellulose, soybean oil and vitamins/minerals/marker) or a 15% gelatin-based diet was fed to growing pigs and ileal digesta was collected at slaughter. One pooled ileal digesta sample was obtained for the protein-free diet by combining samples of two pigs based on the freeze-dry matter content (n = 1). Two pooled ileal digesta samples were obtained for the gelatin diet by combining samples of two and four pigs based on the freeze-dry matter content (n = 2). In the fifth experiment (S. M. Rutherfurd, Riddet Institute, personal communication), diets contained one of two whey protein concentrates or a whey protein isolate as the sole protein source and were fed to growing pigs. Ileal digesta was collected at slaughter. The ileal digesta of three, five and four pigs for the two whey protein concentrate diets and the whey protein isolate diet, respectively, were pooled based on the freeze-dry matter content (n = 2 for whey protein concentrate and n = 1 for whey protein isolate).

Samples from broilers were obtained from two experiments. In the first experiment, maize (30%) and rapeseed meal (25%) were the main protein-containing ingredients in the experimental diet and ileal digesta samples were collected at slaughter. The experimental diet contained crystalline α-Lys HCl. The ileal digesta from six cages containing 11 broilers were pooled based on freeze-dry matter content (n = 2 by pooling samples per three cages). In the second experiment, wheat (65%) and soybean meal (28%) were the main protein-containing ingredients in two experimental diets and ileal digesta samples were collected at slaughter. The experimental diets contained crystalline α-Lys HCl. The ileal digesta of six cages per experimental diet containing eight broilers were pooled based on the freeze-dry matter content (n = 4 by pooling samples per four and two cages per experimental diet).

The samples were analyzed for free Lys and total Lys content using the methods described above. The contribution of endogenous or dietary free Lys to the total free Lys content in ileal digesta was determined by comparing ileal digesta from growing pigs fed protein-free or protein-containing diets. The free Lys content in ileal digesta collected at slaughter from growing pigs or broilers fed protein-containing diets was also compared.

**Calculations.** The recovery of amino acids after OMIU incubation was calculated using eq 1:

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\text{Amino acid recovery (\%)} = \frac{\text{amino acid in OMIU incubated sample (nmol/mg)}}{\text{amino acid in non-OMIU incubated sample (nmol/mg)}} \times 100
\]

For crystalline α-Lys, the difference between Lys in the non-OMIU incubated sample (100% recovery) and the sum of the recovery of unreacted Lys (i.e., Lys having a free α- and ε-amino group), and homoarginine (i.e., Lys with OMIU bound to the ε-amino group), was attributed to Lys with a CN2H3 (i.e., OMIU) bound to the α- and ε-amino group (i.e., Lys that could not be recovered by HPLC analysis). For the other crystalline amino acids, the difference between the recovery of the amino acid in the non-OMIU incubated sample (100% recovery) and the recovery of the amino acid in the OMIU incubated sample was attributed to the amino acid with a CN2H3 (i.e., OMIU) bound to the α-amino group.

The free Lys contents in ileal digesta collected via a cannula or at slaughter of growing pigs fed soybean meal or rapeseed meal were plotted against the apparent ileal digestible crude protein (CP) content of the diets. Correlations between the free and total Lys content in ileal digesta of growing pigs fed protein-containing diets and between the apparent ileal digestible CP content and the free Lys as percentage of total Lys were statistically analyzed using the PROC CORR procedure in SAS 9.3 (SAS Inst. Inc., Cary, NC).

**RESULTS AND DISCUSSION**

**Binding of OMIU to α- and ε-Amino Groups.** As expected, the recovery of unreacted Lys (i.e., having free α- and ε-amino groups) when crystalline i-Lys was incubated with OMIU was low. However, the recovery of homoarginine was low as well, resulting in a significant amount of Lys (i.e., 96%) being unaccounted for after guanidination (Figure 1). The latter was also observed for the other six amino acids (Figure 1). The unrecovered amino acids after incubation with OMIU were expected to have reacted with OMIU via their α-amino groups and the subsequent inability of the compound to be retained on the ion-exchange column or to be derivatized by o-phthalaldehyde after chromatographic separation. The difference between amino acids in terms of recovery of the unreacted amino acid suggests that there may be a different reaction equilibrium for each amino acid, possibly related to differences between side-chains (i.e., charged vs uncharged and polar vs nonpolar) and the pK_a of α-amino groups of the different amino acids. Binding of OMIU both to the α- and ε-amino groups of crystalline i-Lys and to the α-amino group of crystalline i-Tyr was confirmed by MS. After incubation of crystalline i-Lys with OMIU, protonated Lys (1.38 min, m/z 147.11), protonated monodervative Lys/homoarginine (1.8 min, m/z 189.13), and protonated double derivatized Lys (2.9 min, m/z 231.16) were identified (Figure 2A). Furthermore, the ratio of these three compounds was dependent on the OMIU to Lys ratio used for incubation. The m/z values for the peaks at 1.79 min (Figure 2B) and 2.94 min (Figure 2C) are consistent with those of nonprotonated homoarginine (188.23 g/mol) and nonprotonated double derivatized Lys (230.27 g/mol). In addition to the m/z value for the intact molecules, several m/z values corresponding to fragment ions were also visible, such as m/z 172.11 (protonated homoarginine without NH_3) and m/z 213.14 (protonated double derivatized Lys without 2 × H and 1 × O). After incubation of crystalline i-Tyr with OMIU, protonated Tyr (<5.5 min, m/z 182.08) and protonated monodervative Tyr (<6.7 min, m/z 224.10) were identified (Figure 2D). As was the case with Lys, the ratio of these two compounds was dependent on the OMIU to Tyr ratio. The m/z for the peak at 6.70 min (Figure 2E) is...
consistent with those of nonprotonated monoderivatized Tyr (223.22 g/mol). In this case, OMIU reacted only with the α-amino group of Tyr because there is no binding site on the aromatic ring. In peptides, OMIU has been reported to bind to the α-amino group of Gly and partially to the α-amino group of Met, Ser, Val, Leu, Phe, Glu, and Ala when reaction time was extended to several hours. However, since MALDI MS was used, only qualitative results were provided and the extent of the binding of OMIU to α-amino groups could not be determined. The latter along with the fact that different reactions times were used between the study of Beardsley and Reilly (5 or 10 min) and the study reported here (3 or 7 days) make comparison of results difficult. Nonetheless, under the reaction conditions that were employed in the present study, OMIU was found to bind extensively to the α-amino group of several crystalline amino acids. Moreover, the OMIU to amino acid ratio used during incubation appeared to have a major influence on the specificity of OMIU for the ε-amino group of Lys. The effects of OMIU to amino acid ratio, pH of the OMIU solution, and reaction time were subsequently studied to investigate the specificity of OMIU to react with the ε-amino group of crystalline L-Lys.

Optimization of the Guanidination Reaction for Crystalline L-Lys. Regardless of the OMIU to Lys ratio, reaction time had little effect on the recovery of unreacted Lys and homoarginine (Figure 3) and on the quantity of 

Figure 2. LC-MS results of O-methylisourea (OMIU) incubated samples: crystalline L-Lys (A) and crystalline L-Tyr (D) incubated at an OMIU to crystalline L-Lys or crystalline L-Tyr ratio of 10:1, 100:1, and 1000:1, and MS spectra of the LC peaks at 1.79 min (B) and 2.94 min for crystalline L-Lys (C) and at 6.70 min for crystalline L-Tyr (E).

Figure 3. Recovery of crystalline L-Lys after the guanidination reaction using four O-methylisourea (OMIU) to free Lys ratios (1.5:1, 10:1, 100:1, or 1000:1), three reaction times (1, 3, or 7 days), and a pH of the OMIU solution of 10.6. Black bars indicate unreacted Lys, white bars indicate homoarginine, and gray bars indicate nonrecovered Lys.
nonrecovered Lys (considered to be double derivatized Lys). The recovery of unreacted Lys and homoarginine decreased from 9 to 1% and from 51 to 1%, respectively, when the OMIU to Lys ratio increased from 1.5:1 to 1000:1. These results were consistent with the findings obtained using MS analysis (Figure 2A). The impact of the reaction mixture pH on the binding of OMIU to the α- and ε-amino group of crystalline L-Lys was also examined (Figure 4). When the OMIU to Lys ratio was 10:1, the recovery of homoarginine increased from 75 to 99% of the crystalline L-Lys as homoarginine. Moreover, in all cases, between 4 and 99% of the crystalline L-Lys was not recovered either as unreacted Lys or as homoarginine after incubation with OMIU, suggesting that OMIU had bound to the α-amino group of Lys to differing extents. Increasing the amount of OMIU appeared to drive the equilibrium of the chemical reaction toward double derivatization of the crystalline L-Lys. Typically, a higher OMIU to Lys ratio is preferred for the guanidination of protein-bound Lys present in food/feed ingredients and diets, having only a free ε-amino group, in order to completely convert protein-bound Lys to homoarginine. However, if Lys with a free ε-amino group, i.e. crystalline L-Lys, free Lys, or N-terminal Lys, is present in those protein sources or diets, then as the OMIU to Lys ratio is increased, the double derivatization of this Lys also appears to increase. Lowering the OMIU to Lys ratio to 1.5:1, however, resulted in a 51% recovery of homoarginine, indicating that, even at low OMIU to Lys ratios, it is still possible for OMIU to bind to the α-amino group of Lys. These results appear to be in contrast to those of Zhang et al., who reported a conversion of Lys to homoarginine of 99.5% for an OMIU to Lys ratio of 1.5:1. Conversion of Lys to homoarginine in the latter study, however, was calculated as the molar amount of homoarginine divided by the sum of the molar amounts of homoarginine and unreacted Lys. This manner of expressing conversions is often used but does not take into account the conversion of Lys to double derivatized Lys or other Lys derivatives. When applying this equation to the data of the current study, conversions of >90% were found (data not shown). This is in contrast with the low recovery of homoarginine that was actually observed in the present study. Thus, the conversion of Lys to homoarginine can appear to be high while actually a large proportion of Lys is in the double derivatized form. The low recovery of homoarginine could result in an underestimation of the reactive Lys content and subsequently an overestimation of Lys damage. When considering protein-bound Lys to be fully converted to homoarginine, the underestimation of the reactive Lys content in food/feed ingredients and diets depends on the amount of Lys with a free α-amino group (free + N-terminal Lys).

The pH of the OMIU solution clearly affected the guanidination reaction. This reaction depends on the amino group being deprotonated (i.e., pH > pKα) for the reaction with OMIU to occur.23 The pKα of the ε-amino group of Lys is 10.6 and the recovery of homoarginine should be highest when the pH of the OMIU solution is greater than 10.6. The latter was also found in the current study (homoarginine recovery of 75%; Figure 4). The pKα of the α-amino group of Lys is 9.0, and the recovery of unreacted Lys (i.e., no binding of OMIU to either amino group) should be highest when the pH of the OMIU solution is smaller than 9.0. Again, this was found in the current study (average unreacted Lys recovery of 70%; Figure 4). The results are, however, not conclusive with regard to the pH of the OMIU solution, since homoarginine is also recovered at pH values smaller than 10.6. The effect of the pH of the OMIU solution was clearly seen for an OMIU to Lys ratio of 10:1. For an OMIU to Lys ratio of 1000:1, the OMIU pH of 8.6 resulted in a high recovery of unreacted Lys (53%) and an OMIU pH of 9.0 in a high recovery of homoarginine (61%). The excess of OMIU for an OMIU pH greater than 9.0 apparently drove the reaction toward both amino groups, irrespective of protonation/deprotonation. Several authors have reported different optimal pH values of the OMIU solution for different protein sources.2,28–30 The optimal pH for free Lys is approximately 10.6 (Figure 4), but none of the pH values resulted in a 100% recovery of homoarginine.

To confirm the specificity of OMIU for the ε-amino group of crystalline L-Lys, the homoarginine content after incubation with OMIU should be equal to the level of Lys added to the reaction mixture (i.e., complete recovery of Lys as homoarginine). Unfortunately, none of the combinations of reaction time with OMIU to Lys ratio and pH of the OMIU solution with OMIU to Lys ratio used in the present study resulted in specific binding of OMIU to the ε-amino group of crystalline L-Lys. The best reaction conditions (i.e., maximal conversion of crystalline L-Lys to homoarginine and minimal conversion of crystalline L-Lys to double derivatized Lys) were reaction at pH 10.6 for 3 days with an OMIU to Lys ratio of 10:1, which resulted in a homoarginine recovery of 75%. It seems unlikely that the guanidination reaction for free and N-terminal Lys can be optimized to obtain complete conversion of Lys to homoarginine. Moreover, it is also unlikely that both protein-bound and free + N-terminal Lys can be measured using one set of reaction conditions.

**Analysis of Crystalline L-Lys HCl and a Mixture of Crystalline Amino Acids Using Optimized Guanidination Conditions.** The optimized guanidination conditions (pH 10.6, OMIU to amino acid ratio of 10:1 and reaction time of 3 days) were applied to crystalline L-Lys HCl and a mixture of six amino acids (Arg, Phe, Val, Ile, Thr, and Gly) in order to test
the reactivity of the α-amino groups of crystalline L-Lys HCl (a commercially available form of crystalline L-Lys often used as a supplement for pig and poultry diets) and six other amino acids under these guanidination conditions. An OMIU to amino acid ratio of 1000:1 was used, since these conditions have been used previously to determine reactive Lys in food/feed ingredients. Incubating crystalline L-Lys HCl with OMIU resulted in a homoarginine recovery of 19.5 and 1.1% whereas the nonrecoverable Lys was 79 and 98%, respectively, when the OMIU to Lys ratio was either 10:1 or 1000:1, respectively. The recovery of the other six other amino acids (Arg, Phe, Val, Ile, Thr, and Gly) was also low (<26 and <38% for an OMIU to amino acid ratio of 10:1 and 1000:1, respectively) when incubated with OMIU as described above. Again, these results suggest that OMIU can bind to the free α-amino groups not only of crystalline L-Lys but also of crystalline L-Lys HCl and the free α-amino groups of crystalline amino acids other than Lys, irrespective of the reaction conditions used. Specificity of OMIU. The results described above clearly demonstrate that OMIU can react with α-amino groups in addition to the ε-amino group of Lys. Furthermore, none of the reaction conditions used in the present study resulted in the complete guanidination of the ε-amino group of Lys without guanidination of the α-amino group. Thus, it is unlikely that guanidination conditions can be optimized in the future to achieve specificity for Lys with a free α-amino group (free + N-terminal Lys). Previously, authors have reported the recovery of all amino acids after

### Table 1. Free Lys and Total Lys Content (g/kg as-fed basis) and Free Lys as Percentage of Total Lys in 44 Different Food/Feed Ingredients (adapted from Ajinomoto Eurolysine s.a.s.25)

| class               | food/feed ingredient       | number of samples | free Lys content | total Lys content | free Lys as % of Lys |
|---------------------|----------------------------|-------------------|------------------|-------------------|----------------------|
| cereals             | wheat                      | 114               | 0.04             | 3.16              | 1.3                  |
|                     | barley                     | 64                | 0.04             | 3.80              | 1.1                  |
|                     | corn                       | 89                | 0.08             | 2.33              | 3.4                  |
|                     | triticale                  | 29                | 0.02             | 3.63              | 0.6                  |
|                     | oats                       | 4                 | 0.03             | 4.91              | 0.6                  |
|                     | rice                       | 4                 | 0.04             | 3.03              | 1.3                  |
|                     | rye                        | 4                 | 0.03             | 3.26              | 0.9                  |
|                     | sorghum                    | 2                 | 0.02             | 2.16              | 0.9                  |
| cereal byproducts   | wheat middlings and bran   | 23                | 0.09             | 5.94              | 1.5                  |
|                     | wheat gluten               | 15                | 0.05             | 12.51             | 0.4                  |
|                     | wheat gluten feed          | 4                 | 0.07             | 5.48              | 1.3                  |
|                     | wheat DDGS                 | 44                | 0.05             | 6.40              | 0.8                  |
|                     | corn feed flour            | 2                 | 0.13             | 4.05              | 3.2                  |
|                     | corn gluten meal 60% CP    | 16                | 0.21             | 10.03             | 2.1                  |
|                     | corn germ                  | 2                 | 0.48             | 8.26              | 5.8                  |
|                     | corn DDGS                  | 6                 | 0.06             | 7.47              | 0.8                  |
|                     | rice protein               | 3                 | 0.02             | 18.94             | 0.1                  |
| vegetable protein sources | soybean meal              | 132               | 0.14             | 28.41             | 0.5                  |
|                     | full fat soybean           | 37                | 0.16             | 22.43             | 0.7                  |
|                     | soy protein concentrate S2–56% CP | 21 | 1.28 | 32.55 | 3.9 |
|                     | soy protein concentrate 65% CP | 13 | 0.12 | 40.96 | 0.3 |
|                     | rapeseed meal              | 43                | 0.06             | 18.60             | 0.3                  |
|                     | full fat rapeseed          | 2                 | 0.07             | 12.16             | 0.6                  |
|                     | sunflower meal 28% CP      | 14                | 0.18             | 10.13             | 1.8                  |
|                     | sunflower meal 33% CP      | 9                 | 0.12             | 11.54             | 1.0                  |
|                     | sunflower meal 37% CP      | 7                 | 0.26             | 13.82             | 1.9                  |
|                     | palm kernel meal           | 3                 | 0.00             | 3.75              | 0.0                  |
|                     | fava bean                  | 2                 | 0.10             | 17.39             | 0.6                  |
|                     | lupin seed                 | 10                | 0.22             | 16.27             | 1.4                  |
|                     | pea                        | 22                | 0.12             | 14.78             | 0.8                  |
|                     | potato protein concentrate | 24                | 0.16             | 61.86             | 0.3                  |
| dairy products      | milk                       | 23                | 0.11             | 18.94             | 0.6                  |
|                     | whey powder                | 71                | 0.09             | 9.89              | 0.9                  |
|                     | whey protein concentrate   | 10                | 0.05             | 30.27             | 0.2                  |
| miscellaneous       | fish meal                  | 51                | 0.89             | 54.01             | 1.6                  |
|                     | blood meal                 | 2                 | 0.02             | 79.02             | 0.0                  |
|                     | feather meal               | 5                 | 0.17             | 19.69             | 0.9                  |
|                     | poultry protein            | 3                 | 0.49             | 33.28             | 1.5                  |
|                     | plasma                     | 4                 | 0.08             | 65.02             | 0.1                  |
|                     | egg                        | 5                 | 0.92             | 45.64             | 2.0                  |
|                     | cassava                    | 2                 | 0.03             | 0.95              | 3.2                  |
|                     | brewers’ yeast             | 7                 | 1.11             | 27.36             | 4.1                  |
|                     | bakery byproducts          | 5                 | 0.03             | 2.63              | 1.1                  |

“Determined after acid hydrolysis in 6 M HCl at 110 °C for 24 h. DDGS = distillers dried grain with solubles. CP = crude protein.”
guanidination to approximate 100% for lysozyme, soy protein isolate, skim milk powder, lactic casein, whey protein concentrate, soy protein concentrate, blood meal, and cottonseed meal.\(^\text{12}\) This suggests that the level of free + N-terminal Lys in these ingredients is low. The free Lys content in 44 different food/feed ingredients was compiled and found to range from 0 to 5.8% of total Lys, with an average of 1.3% (Table 1). Consequently, the underestimation of the OMIU-reactive Lys content for these food/feed ingredients is expected to be low. The estimates of the OMIU-reactive Lys content are expected to be inaccurate only in those cases where the test material contains a large proportion of free + N-terminal Lys. Materials for which OMIU-reactive Lys estimates could be inaccurate are materials that contain crystalline i-Lys (e.g., practical pig and poultry diets, enteral nutrition formula, and specific pet foods), hydrolyzed products (e.g., hydrolyzed feather meal, hydrolyzed vegetable protein, infant formula, hypoallergenic diets), and potentially digested obtained from the small intestine.

In order to determine the potential error involved in the measurement of reactive Lys in ileal digesta samples, 23 nonpooled and seven pooled ileal digesta samples from growing pigs and six pooled ileal digesta samples from broilers were analyzed for their free and total Lys content. The free Lys as a percentage of total Lys for two samples from growing pigs (one from a protein-free diet and the other from a soybean meal diet) were considered outliers (\(\text{mean} = -2 \times \text{SD}\)) and were, therefore, excluded from the data analysis. The mean (±SD) free and total Lys contents across the remaining 34 ileal digesta samples from growing pigs and broilers fed protein-free and protein-containing diets were 0.74 (±0.39) and 5.74 (±2.49) g/kg as-is, respectively. The free Lys, therefore, was on average 12.8% of the total Lys present in the ileal digesta. This amount was unexpectedly high considering that trypsin cleaves at the carboxyl terminal of Lys.\(^{32}\) Multiple carrier transport systems are involved in the absorption of different amino acids, and absorption rates differ between amino acids. For example, Thr and branched-chain amino acids (Leu, Ile, and Val) are rapidly absorbed while Lys and Arg are more slowly absorbed.\(^{33}\) Moreover, peptides use a different carrier transport system from that used by amino acids\(^{34}\) and are absorbed more rapidly than free amino acids.\(^{35}\) A slow absorption rate of Lys and a preference for the absorption of peptides might explain the relatively large amount of free Lys present in the ileal digesta. Of the Lys in ileal digesta collected from growing pigs fed a protein-free diet or a protein-containing diet, 13.0 or 12.7% was free Lys (Figure 5A). Asche et al.\(^{36}\) reported that approximately 20% of proteinaceous material in the soluble fraction of ileal digesta of growing pigs fed a protein-free diet had a molecular weight less than 1000 Da (considered to consist of free amino acids and small peptides) while for a corn-soybean meal diet the equivalent value was approximately 13%. Unfortunately, the individual free amino acids were not determined. Zebrowska et al.\(^{37}\) reported that endogenous proteins are absorbed at a slower rate compared to dietary proteins, resulting in an increased concentration of endogenous proteins at the end of the ileum. The data of the current study, however, indicate that the presence of free Lys in ileal digesta is not related to the presence of protein-containing ingredients in the diet. Moreover, the free Lys as a percentage of total Lys in ileal digesta of growing pigs fed SBM or RSM diets was independent (\(R^2 = 0.01, P = 0.71\)) of the apparent ileal digestible CP content in the diet and of collection method (Figure 5B). There appeared to be no difference in the free Lys as a percentage of total Lys between ileal digesta samples collected from growing pigs or broilers (12.7 and 14.4%, respectively; Figure 5A), suggesting that the free Lys content in ileal digesta is not species specific. The amount of free Lys at the terminal ileum of growing pigs and broilers fed protein-containing diets, in the current study, was much higher than the 3.1% reported by Moughan and Schuttert.\(^{37}\) This may be due to the relatively slower absorption of free Lys compared with peptides\(^{38}\) or spontaneous nonenzymatic breakdown of peptides due to their instability after hydrolysis.\(^{38}\) The thawing of fresh samples for subsampling might also have affected the level of free Lys in ileal digesta, but this effect is expected to be low. Separating pig ileal digesta by centrifugation (14,500 relative centrifugal force for 30 min at 4 °C) resulted in the separation of porcine and microbial cells (precipitate) from soluble proteins, peptides, free amino acids, and mucins (supernatant). Approximately half of the protein present in the supernatant was of microbial origin. While the microbial cells are most likely to be present in the precipitate, the supernatant might contain free Lys.

![Figure 5](image-url)
originating from lysed microbial cells. This source of free Lys might also have added to the free Lys content in pig ileal digesta analyzed in the current study. There was a linear relation between the total and free Lys contents in ileal digesta of growing pigs fed protein-containing diets ($R^2 = 0.54$, $P < 0.001$; Figure 5C). Therefore, the methodology of determining free amino acids which involves the use of 0.1 M HCl coextraction of nitrogenous macromolecules by sulfosalicylic acid may have hydrolyzed Lys from peptides or proteins, thereby, overestimating the free Lys content relative to that present in digesta at the terminal ileum. The latter may explain the lower value reported by Moughan and Schuttert, as these authors used a different methodology to determine free amino acids in ileal digesta of pigs fed protein-free diets.

The impact of the nonspecificity of OMIU and the free Lys content in ileal digesta on the standardized ileal digestibility of OMIU-reactive Lys was assessed using samples from a previous study. The standardized ileal digestibility was calculated considering supplemented dietary crystalline L-Lys HCl to be completely absorbed from the small intestine before the terminal ileum in growing pigs. Moreover, it was assumed that all free Lys in ileal digesta was double derivatized and, therefore, not determined as OMIU-reactive Lys. For the soybean meal and rapeseed meal ingredients examined, the determined standardized ileal OMIU-reactive Lys digestibilities were 92.8 and 83.5%, respectively, and the standardized ileal digestible OMIU-reactive Lys content was 5.6 and 4.2 g/100 g CP, respectively. The equivalent recalculated values for the soybean meal and rapeseed meal ingredients examined, the determined standardized ileal OMIU-reactive Lys digestibilities were 92.8 and 83.5%, respectively, and the standardized ileal digestible OMIU-reactive Lys content was 5.6 and 4.2 g/100 g CP, respectively. The overestimation will be greater if ileal digesta contains a significant amount of peptides containing a N-terminal Lys residue.

In conclusion, OMIU was found to be not specific for the ε-amino group of crystalline L-Lys (HCl) and able to bind to the α-amino groups of crystalline amino acids under the reaction conditions of the assay as developed by Moughan and Rutherford. The various guanidination conditions of the OMIU-reactive lysine assay investigated did not result in absolute specificity for the ε-amino group of Lys. It is recommended to analyze the reactive Lys content of food/feed ingredients, diets and ileal digesta using an OMIU pH of 10.6, an OMIU to Lys ratio of 1000:1, and a reaction time of at least 3 days to fully convert protein-bound Lys to homoaarginine. These samples should subsequently be analyzed for their free Lys content to calculate the reactive Lys content of the samples (i.e., assuming free Lys to be 100% reactive). The accurate quantification of free and N-terminal amino acids in ileal digesta warrants further investigation as well as the search for a reagent which is specific for the ε-amino group of Lys.

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The authors declare no competing financial interest.

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### Abbreviations Used
CP, crude protein; OMIU, O-methylisourea; UPLC, ultrahigh-performance liquid chromatography

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