Contribution of purified soybean trypsin inhibitor and exogenous protease to endogenous amino acid losses and mineral digestibility

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ABSTRACT The primary objective of the current study was to evaluate the impact of trypsin inhibitor (TI) and exogenous protease supplementation on endogenous loss of amino acids (AA) in broiler chickens. A total of 384 Cobb-500 broiler chicks were allocated to 4 nitrogen-free diets, each with 8 replicate cages and 12 birds per replicate. The diets were arranged as a 2 × 2 factorial with factors being dietary TI (0 or 8,000 TIU/g) and exogenous protease (0 or 15,000 PROT/kg). Desired dietary TI concentration was achieved by addition of commercially available, purified soybean TI. There was no effect of TI or exogenous protease or their interaction on growth performance of birds. However, the endogenous loss of nitrogen (N) and all AA increased (P < 0.05) due to dietary TI concentration except for Cys. The increase in endogenous AA due to TI ranged from 17% for Thr to 52.2% for Trp. Exogenous protease had no effect on endogenous loss of N and all AA. There was no effect of TI or exogenous protease or their interaction on the AID of P, however AID of Ca, Fe, Mg, Mn, and Cu was reduced (P < 0.05) due to dietary TI. The AID of Cu (P < 0.01) and K (P < 0.05) improved with exogenous protease supplementation. Significant interactions (P < 0.05) between exogenous protease and TI existed for Zn, Mg, Cu, and Na. The concentration (g/kg DM intake) of crude mucin and sialic acid increased (P < 0.05) with increased dietary TI. Decreased trypsin (P < 0.001) and increased chymotrypsin (P < 0.001) activity in the pancreas were observed as a result of exogenous protease supplementation. In conclusion, the current study showed that TI increases the endogenous loss of AA and reduced the digestibility of minerals in broiler chickens. Furthermore, exogenous protease did not affect endogenous AA flow, irrespective of added purified dietary TI.

Key words: amino acid, endogenous loss, mineral, protease, trypsin inhibitor

INTRODUCTION

During the digestive process in the gastrointestinal tract, endogenous proteins from digestive secretions (saliva, bile, gastric, and pancreatic secretions as well as intestinal secretions), mucoproteins, sloughed intestinal epithelial cells, serum albumin, and amides (Nyachoti et al., 1997; Ravindran and Bryden, 1999) are continuously secreted into the lumen of the intestine. A previous estimate (Nasset and Ju, 1961) reveals that endogenous protein secretion may be five times as abundant as those of dietary origin and, about 60 to 79% of this gross endogenous secretion may be reabsorbed (Krawielitzki et al., 1990; Souffrant et al., 1993). However, the degree of reabsorption will vary depending on the relative ratio of individual endogenous components and their point of entry into the gut (Souffrant, 1991). Regardless of the intestinal site, net endogenous losses are the result of total secretion, reabsorption, and reutilization of reabsorbed endogenous protein. The unabsorbed portion is inevitably lost to the animal and referred to as basal (or diet-independent) endogenous losses, representing the amino acids that are lost irrespective of whether the animal is fed protein.

The level of endogenous amino acid (EAA) flow may also depend on the presence of antinutritive factors in the gut (Barth et al., 1993; Schulze, 1994). Schulze (1994) studied the effect of various inducing agents such as trypsin inhibitors (TI), lectins, and fiber, on EAA flow at the terminal ileum in pigs and found that endogenous nitrogen (N) secretions increased by 7.3, 5.2, and 0.04 g per g lectins, TI, and fiber respectively. For TI and fiber, there were linear increases in EAA flow with increasing amounts of the inducing agent (Schulze, 1994). While soybean meal (SBM) provides...
an excellent source of protein for nonruminants, it contains variable amounts of TI depending on processing conditions (Newkirk, 2010). This may lead to increased TI intake which affects dietary protein digestion and amino acid utilization in nonruminants by inhibiting the activation of the proteolytic enzymes in the gut (Rawlings et al., 2004; Erdaw et al., 2017). On the other hand, exogenous protease supplementation is a widely accepted strategy to improve dietary protein utilization in nonruminants (Clarke and Wiseman, 2005; Erdaw et al. 2017; Aderibigbe et al., 2020). Additionally, exogenous protease has been shown to improve EAA recovery in birds (Cowieson and Roos, 2016). Likewise, an in vitro trial (Huo et al., 1993) showed that exogenous protease has been shown to improve EAA (Table 1) in a randomized complete block design, with BW as a blocking factor, resulting in 8 blocks per treatment. Each diet comprised of 8 replicates cages and 12 birds per replicate cage. The TI used in the current study is a commercially available purified form of soybean TI (Sigma Aldrich, St. Louis, MO; EC 2329069). Titanium dioxide was included as an indigestible marker.

There are limited number of studies that report the direct impact of TI on EAA flow, and to the best of our knowledge, these have not been reported for broiler chickens. Therefore, the hypothesis of the current study was that TI and exogenous protease will affect the loss of endogenous protein in broiler chickens. To test this hypothesis, specific objectives were to 1) determine the contribution of purified soybean TI and exogenous protease to EAA flow and mineral digestibility in broiler chickens fed nitrogen-free diet (NFD); 2) evaluate the effect of exogenous protease in diets containing purified soybean TI on EAA flow and mineral digestibility in broiler chickens fed NFD.

### MATERIALS AND METHODS

Protocols of animal experiments were reviewed and approved by the Purdue University Animal Care and Use Committee.

### Experimental Diets and Birds

A total of 384 male 0-d-old Cobb 500 broiler chicks (Cobb-Vantress, Siloam Springs, AR) were procured from a commercial hatchery. Birds were individually tagged, weighed, raised in heated battery brooders (model SB 4 T; Alternative Design Manufacturing, Siloam Springs, AR) and fed a nutritionally adequate corn-SBM based diet (210 g/kg CP; 3,100 kcal/kg ME) until d 20 post hatching. Temperature and lighting were maintained as previously described by Aderibigbe et al. (2020). Twelve hours prior to experimental diet allocation, birds were fasted to empty the gut of residual dietary N. Subsequently on d 21 post hatching, using a 2 × 2 factorial arrangement, with 2 concentrations of dietary TI (0 or 8,000 TIU/g) and exogenous protease (0 or 15,000 PROT/kg; Ronozyme ProAct, DSM Nutritional Products, Kaiseraugst, Switzerland) the chicks were allotted to 4 experimental NFD (Table 1) in a randomized complete block design, with BW as a blocking factor, resulting in 8 blocks per treatment. Each diet comprised of 8 replicates cages and 12 birds per replicate cage. The TI used in the current study is a commercially available purified form of soybean TI (Sigma Aldrich, St. Louis, MO; EC 2329069). Titanium dioxide was included as an indigestible marker.

### Sampling Procedures

Birds were fed experimental diets on an ad libitum basis for a 3-d period, and mortality records was taken daily. On d 24 post hatching, feed intake was measured per cage and birds were individually weighed. Gain to feed ratio was calculated and corrected for the body weight of any bird that died or was culled during the experimental period. Subsequently, all birds were euthanized by CO₂ asphyxiation and eviscerated for sample collection. Ileal digesta was collected from the distal two-thirds of the section of the ileum between the Meckel’s diverticulum and approximately 2-cm cranial to the ileocecal junction. The ileal digesta was pooled within

### Table 1. Ingredient and calculated nutrient composition of experimental diets, as-fed basis.

| Ingredient(s) | 0 | 15,000 |
|--------------|---|--------|
| Purified trypsin inhibitor, TIU/g: | 0 | 8,000  | 0 | 8,000 |
| **Protease, PROT/kg:** | | | | |
| 0 | 15,000 |
| **Ingredients, g/kg:** | | | | |
| Dextrose | 615.5 | 565.5 | 605.5 | 555.5 |
| Corn | 200.5 | 200.5 | 200.5 | 200.5 |
| Soybean oil | 50.0 | 50.0 | 50.0 | 50.0 |
| Solka floc | 50.0 | 50.0 | 50.0 | 50.0 |
| Ground limestone | 15.5 | 15.5 | 15.5 | 15.5 |
| Monocalcium phosphate | 21.0 | 21.0 | 21.0 | 21.0 |
| Potassium carbonate | 2.6 | 2.6 | 2.6 | 2.6 |
| Magnesium oxide | 2.0 | 2.0 | 2.0 | 2.0 |
| Sodium bicarbonate | 7.5 | 7.5 | 7.5 | 7.5 |
| Choline chloride | 2.5 | 2.5 | 2.5 | 2.5 |
| Potassium chloride | 2.9 | 2.9 | 2.9 | 2.9 |
| Vitamin-mineral premix | 5.0 | 5.0 | 5.0 | 5.0 |
| Trypsin inhibitor premix | 0.0 | 0.0 | 0.0 | 0.0 |
| Ronozyme ProAct premix | 0.0 | 0.0 | 0.0 | 0.0 |
| Titanium dioxide premix | 25.0 | 25.0 | 25.0 | 25.0 |
| Total | 1,000 | 1,000 | 1,000 | 1,000 |
| **Calculated composition:** | | | | |
| ME kcal/kg | 3,606 | 3,606 | 3,606 | 3,606 |
| Crude protein, g/kg | 6.0 | 6.0 | 6.0 | 6.0 |
| Ether extract, g/kg | 48.50 | 48.50 | 48.50 | 48.50 |
| Ca, g/kg | 9.10 | 9.10 | 9.10 | 9.10 |
| P, g/kg | 4.52 | 4.52 | 4.52 | 4.52 |
| DEB, mEq/kg diet | 90.98 | 90.98 | 90.98 | 90.98 |
| **Analytical composition:** | | | | |
| Crude protein, g/kg | 1.16 | 3.15 | 1.24 | 2.58 |
| Trypsin inhibitor, TIU/g | 6,244 | 6,244 | 6,244 | 6,244 |

138% Ca, 16% Ca, 21% P.

61 g of Titanium dioxide added to 4 g of dextrose.

6Supplied the following per kg diet: vitamin A, 8,575 IU; vitamin D₃, 4,300 IU; vitamin E, 28.58 IU; menadione, 7.30 mg; riboflavin, 9.15 mg; \( \alpha \) - pantothenic acid, 18.33 mg; niacin, 73.5 mg; choline chloride, 1,285 mg; vitamin B₁₂, 0.02 mg; biotin, 0.09 mg; thiamine mononitrate, 3.67; folic acid, 1.65 mg; pyridoxine hydrochloride, 5.50 mg; I, 1.85 mg; Mn, 178.5 mg; Cu, 7.40 mg; Fe, 73.5 mg; Zn, 178.5 mg.

8Purified trypsin inhibitor (PTI) from soybeans product contains 9,000,000 TIU/g. 1 g of STI added to 55.25 g of corn supplies 160,000 TIU/g of premix. 50 g/kg premix delivers 15,000 PROT/kg feed.

91 g of Titanium dioxide added to 4 g of dextrose.

9Dietary electrolyte balance (mEq/kg diet) was calculated as Na\(+\)-K\(-\).
Digestive Enzyme, Crude Mucin, and Sialic acid Assays

The pancreas was collected from 2 median bird per replicate cage, frozen in liquid nitrogen and stored at −80°C until required for enzyme assay. Enzymes activities were determined as previously described by Adelibige et al. (2020) using a commercially available assay kit (Sigma Chemical Co, St. Louis, MO). Crude mucin and sialic acid concentration were determined based on methods previously described by Horn et al. (2009). Briefly, excreta samples were freeze-dried for 96 h and subsequently ground to pass through a 0.5-mm screen (Retsch ZM 100, GmbH, Haan, Germany). Subsequently, 3 g of the lyophilized excreta sample was placed in a 50-mL plastic centrifuge tube. Twenty milliliters of chilled NaCl solution (0.15 M NaCl, 0.02 NaNO₃, kept at 4°C) was added to the excreta sample and homogenized for 30 s (T25 Basic, IKA Corp., Staufen, Germany). The mixture was then centrifuged at 12,000 × g for 20 min at 4°C, and the soluble supernatant was decanted into a 50-mL pre-weighted tube. For extraction of mucin proteins, 15 mL of chilled supernatant was decanted into a 50-mL pre-weighed tube. With a mixture of 10 mL of chilled NaCl solution and 15 mL of absolute ethanol was added to the supernatant and allowed to sit overnight at −20°C. The mixture was then centrifuged at 1,400 × g for 10 min at 4°C and the mucin pellet was retained. The mucin pellet was washed with a mixture of 10 mL of chilled NaCl solution (0.15 M NaCl, 0.02 NaNO₃, kept at 4°C) and 15 mL of chilled absolute ethanol and the sample was extracted overnight at −20°C. Subsequently, the mixture was then centrifuged at 1,400 × g for 10 min at 4°C and the wash-extraction cycle was repeated until a clear supernatant was obtained. Water was removed from the mucin pellet by suction and the pellet was then weighed to obtain crude mucin yield. The pellet was then dissolved in 2 mL of distilled water and immediately frozen at −40°C.

Sialic acid concentration was determined from the purified crude mucin samples. Briefly, 100 μL of crude mucin solution was diluted with 100 μL of distilled water in a 1.5-mL microcentrifuge tube and 200 μL of Bial reagent (Ward’s Science, Rochester, NY) was added to the samples and heated for 15 min at 100°C in a water bath (Precision, GCA Corp., College Park, MD). The samples were cooled in room temperature water for 5 min. One mL of isoamyl alcohol (Sigma Chemical Co) was added to the sample, vigorously vortexed, and chilled on ice for 5 min. The samples were then centrifuged at 1,200 × g for 1 min and 200 μL of the upper color phase was gently transferred to a 96-well plate. Absorbance was measured at 560 nm on a UV spectrophotometer (Spark 10M, TECAN, Baldwin Park, CA). Sialic acid concentration was determined from regression of the standard (N-acetyltetraamuramic acid; Sigma Chemical Co, St. Louis, MO) vs. absorbance.

Chemical Analyses and Calculations

Ileal digesta were freeze-dried for 96 h and subsequently ground to pass through a 0.5-mm screen (Retsch ZM 100, GmbH). The dry matter (DM) content of diets and digesta were analyzed by drying overnight at 105°C (Precision Scientific Co., Chicago, IL; method 934.01; AOAC, 2006). Nitrogen content was determined by combustion using a LECO FP-428 nitrogen analyzer (LECO Corp., St. Joseph, MI) with EDTA as a calibration standard. Samples for amino acid (AA) analysis were prepared using a 24-h hydrolysis in 6 N hydrochloric acid at 110°C under an atmosphere of N. Samples were oxidized in performic acid prior to acid hydrolysis for methionine and cysteine analyses. Samples for tryptophan analysis were hydrolyzed using barium hydroxide. Amino acids in hydrolysates were determined by cation-exchange chromatography coupled with post-column derivatization (AOAC, 2000; method 982.30 E [a, b, c]). Following wet digestion using a mixture of nitric and perchloric acid, concentrations of Ca, Fe, Zn, Mg, Mn, Cu, Na, and K in diets and lyophilized ileal digesta samples was determined with appropriate standards using an atomic absorption spectrometer (AAnalyst 300; Perkin Elmer, Norwalk, CT; method 985.01; AOAC, 2006). Concentration of P was determined by spectrophotometry at 620 nm (Spectra Count, model AS1000, Packard, Meriden, CT; AOAC, 2006). Titanium concentration was measured on a UV spectrophotometer (Spark 10M, TECAN) following the method of Short et al. (1996).

The index method was used to calculate the endogenous flow of N and AA in the ileal digesta, crude mucin, and sialic acid in the excreta and apparent ileal digestibility (AID) of macro and trace minerals, according to the following equations:

\[ \text{Endogenous loss, g/kg DM intake} = P_o \times (T_{i}/T_{io}) \]

\[ \text{AID, } \% = 100 - \left[ (T_{i}/T_{io}) \times (Q_o/Q_i) \right] \times 100 \]

where \( T_i \) and \( T_{io} \) are the respective titanium concentrations (g/kg DM) in experimental diets and output (ileal digesta or excreta); \( P_o \) is the concentration of N, AA, crude mucin, or sialic acid (g/kg DM) in the ileal digesta or excreta; \( Q_i \) and \( Q_o \) are the respective concentrations (g/kg DM) of macro and trace minerals in experimental diets and excreta.

Ileal endogenous energy flow (IEEF) was calculated according to the following equations:

\[ \text{IEEF, kcal/kg DM intake} = \left( F_{aa} \times G_{aa} \right)/1000 \]

where \( F_{aa} \) is the endogenous flow of AA (g/kg DM intake) in the ileal digesta and \( G_{aa} \) is the gross energy of individual AA (kcal/kg) adapted from Boisen and Verstegen (2000).

Statistical Analyses

Data were analyzed as a randomized complete block design using the GLM procedures of SAS (SAS Inst.
Inc., Cary, NC). Initial body weight was used as the blocking criterion. Block was treated as a random factor. The main effects of TI and exogenous protease and the interaction were tested accordingly and an α level of 0.05 was considered significant. Where interactions exist, Tukey’s mean separation test was used to make pairwise comparisons.

RESULTS

Overall, there were 11 mortalities during the 3-d trial period, and on-site postmortem examinations did not reveal any direct link to specific dietary treatment. Although the mortality was relatively high, the exact cause was unclear, and is more likely related to the non-physiological nature of the NFD. The effect of purified TI and exogenous protease on growth performance of broiler chickens NFD are shown in Table 2. There were no effects of TI or exogenous protease or their interaction on growth performance of birds. As shown in Table 3, there was no interaction between TI and exogenous protease on EAA loss in birds. Exogenous protease had no effect on endogenous loss of N and all AA. The calculated energetic losses associated with the endogenous protein flows are presented in Table 4. Similar to AA, the ingestion of TI increased \((P < 0.05)\) the energy loss associated with EAA loss, except Cys and exogenous protease addition did not reduce energy loss in EAA.

Table 2. Growth performance of broiler chickens fed nitrogen-free diets supplemented with protease (PROT/kg) and purified trypsin inhibitor (TIU/g), from d 21 to 24 post hatching.

| Item | 0 | 15,000 |
|------|----|--------|
|      | TI | TI     | SEM  | TI | TI | SEM  | P-value | P × TI |
| D 21 BW, g | 631.6 | 631.2 | 631.7 | 631.6 | 0.44 | 631.4 | 631.7 | 631.4 | 0.614 | 0.614 | 0.664 |
| D 24 BW, g | 550.1 | 550.8 | 551.1 | 552.7 | 4.09 | 550.4 | 551.9 | 550.6 | 551.8 | 0.723 | 0.776 | 0.903 |
| Feed intake, g | 81.2 | -81.3 | -80.6 | -79.2 | 4.07 | -81.2 | -79.9 | -80.9 | -80.3 | 0.751 | 0.872 | 0.863 |
| G:F, g/kg | 405.4 | 407.3 | 401.9 | 408.5 | 0.751 | 0.872 | 0.863 |
| N | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8

1 Abbreviations: G:F, gain to feed ratio; P, protease; TI, trypsin inhibitor.

Table 3. Ileal endogenous flow (mg/kg DMI) of nitrogen and amino acid in broiler chickens fed nitrogen-free diets supplemented with exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g), at d 24 post hatching.

| Item | 0 | 15,000 |
|------|----|--------|
|      | TI | TI     | SEM  | TI | TI | SEM  | P-value | P × TI |
| Nitrogen | 1,975 | 2,215 | 1,725 | 2,341 | 157.5 | 2,095 | 2,033 | 2,278 | 0.699 | 0.013 | 0.244 |
| Arg | 434 | 575 | 376 | 607 | 44.5 | 505 | 492 | 405 | 591 | 0.773 | <0.001 | 0.325 |
| His | 208 | 265 | 188 | 280 | 17.6 | 235 | 235 | 199 | 271 | 0.995 | <0.001 | 0.310 |
| Ile | 523 | 622 | 452 | 669 | 43.2 | 573 | 561 | 487 | 646 | 0.787 | 0.001 | 0.185 |
| Leu | 791 | 960 | 681 | 1,019 | 47.9 | 876 | 849 | 736 | 989 | 0.701 | 0.001 | 0.217 |
| Lys | 414 | 552 | 388 | 591 | 47.9 | 483 | 490 | 401 | 571 | 0.890 | 0.002 | 0.499 |
| Met | 146 | 169 | 119 | 190 | 43.2 | 158 | 155 | 133 | 179 | 0.851 | 0.004 | 0.120 |
| Phe | 461 | 569 | 399 | 596 | 40.7 | 515 | 497 | 429 | 583 | 0.675 | 0.001 | 0.290 |
| Thr | 800 | 908 | 747 | 973 | 47.9 | 884 | 860 | 804 | 941 | 0.713 | 0.045 | 0.179 |
| Trp | 74 | 102 | 65 | 108 | 10.4 | 88 | 87 | 69 | 105 | 0.885 | 0.003 | 0.491 |
| Val | 887 | 982 | 743 | 1,044 | 73.2 | 934 | 893 | 815 | 1,013 | 0.585 | 0.013 | 0.174 |

1 Abbreviations: AA, amino acid; P, protease; TI, trypsin inhibitor.
Table 4. Calculated ileal endogenous energy flow (kcal/kg DMI) associated with endogenous amino acids in broiler chickens fed nitrogen-free diets supplemented with exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g), at d 24 post hatching.

| Item | Protease | 0 | 8,000 | 15,000 | SE | Protease | 0 | 15,000 | 0 | 8,000 | SE | Protease | TI | P × TI |
|------|----------|---|-------|--------|----|----------|---|---------|---|-------|----|----------|----|--------|
| Arg  |          | 2.2 | 2.94  | 1.93  | 3.11 | 0.227 | 2.58 | 2.52   | 2.07 | 3.02 | 0.773 | <0.001 | 0.325 |
| His  |          | 1.08 | 1.36  | 0.98  | 1.45 | 0.091 | 1.22 | 1.22   | 1.02 | 1.40 | 0.993 | 0.001 | 0.310 |
| Ile  |          | 3.45 | 4.11  | 2.98  | 4.42 | 0.285 | 3.78 | 3.70   | 3.21 | 4.26 | 0.787 | 0.001 | 0.185 |
| Leu  |          | 5.29 | 6.33  | 4.49  | 6.72 | 0.439 | 5.78 | 5.61   | 4.86 | 6.53 | 0.701 | 0.001 | 0.217 |
| Lys  |          | 2.33 | 3.10  | 2.18  | 3.32 | 0.269 | 2.71 | 2.75   | 2.25 | 3.21 | 0.890 | 0.002 | 0.499 |
| Met  |          | 0.65 | 0.75  | 0.53  | 0.85 | 0.065 | 0.70 | 0.69   | 0.59 | 0.80 | 0.851 | 0.004 | 0.120 |
| Phe  |          | 3.11 | 3.84  | 2.69  | 4.02 | 0.275 | 3.47 | 3.35   | 2.90 | 3.93 | 0.607 | 0.001 | 0.290 |
| Thr  |          | 3.54 | 3.73  | 3.07  | 4.08 | 0.264 | 3.63 | 3.54   | 3.30 | 3.87 | 0.713 | 0.045 | 0.179 |
| Trp  |          | 0.49 | 0.67  | 0.43  | 0.71 | 0.068 | 0.58 | 0.57   | 0.46 | 0.69 | 0.885 | 0.003 | 0.491 |
| Val  |          | 5.29 | 5.87  | 4.44  | 6.24 | 0.437 | 5.58 | 5.34   | 4.87 | 6.05 | 0.585 | 0.013 | 0.174 |

1 Calculated using the flow of each AA in Table 3 and the gross energy of AA adapted from Boisen and Verstegen (2000). Gross energy estimates (kcal/kg) for individual AA from Boisen and Verstegen (2000) are as follows: Arg, 5,115; His, 5,186; Ile, 6,597; Leu, 6,597; Lys, 5,617; Met, 4,446; Phe, 6,740; Tyr, 2.19; His, 5,115; Leu, 6,597; Lys, 5,617; Met, 4,446; Phe, 6,740; Tyr, 2.19; His, 5,115; Leu, 6,597; Lys, 5,617; Met, 4,446; Phe, 6,740; Tyr, 2.19.  
2 Abbreviations: AA, amino acid; P, protease; TI, trypsin inhibitor.

...the direct contribution of TI to EAA losses in broiler chickens, and whether this is affected by exogenous protease supplementation. The impact of soybean TI on growth performance and nutrient digestibility in broiler chickens is widely reported (Clarke and Wiseman, 2005; Erdaw et al., 2017; Aderibigbe et al., 2020). Currently, there are no published data on the effect of TI on pancreas weight was unaffected by TI or exogenous protease. Exogenous protease resulted in a reduction in trypsin activity (P < 0.001) but increased pancreatic chymotrypsin activity (P < 0.001).

**DISCUSSION**

The primary objective of this study was to evaluate the direct contribution of TI to EAA losses in broiler chickens, and whether this is affected by exogenous protease supplementation. The impact of soybean TI on growth performance and nutrient digestibility in broiler chickens is widely reported (Clarke and Wiseman, 2005; Erdaw et al., 2017; Aderibigbe et al., 2020). Currently, there are no published data on the effect of TI on...
endogenous secretion in birds. Because NFD is a commonly used assay for estimating EAA flow in broiler nutrition, we also evaluated the digestibility of macro and trace minerals in an experimental NFD and whether or not this is affected by TI concentration. Our data showed that growth performance of birds was unaffected or not this is affected by TI concentration. This is not surprising because all the birds were fed NFD, which is not expected to support tissue accretion. Compared to conventional diets, feeding NFD, irrespective of protease or TI addition, resulted in accretion. Compared to conventional diets, feeding NFD is especially crucial because variances in feed intake will inevitably at the ileal level if mucus secretion or desquamation is significant, which particularly occurs more distal to the duodenum.

In the current study, and similar to previous observations (Adedokun et al., 2007), 4 AA (Glu, Asp, Ser, and Thr) were predominantly identified in endogenous AA, which could represent up to 34% of total AA flow in the broiler fed an NFD (Adedokun et al., 2007). In the current study, the average increase in EAA loss attributable to TI was 32.3% and was more apparent in Trp (52.2%), Arg (45.9%), and Lys (42.4%). Generally, the

### Table 6. Effect of exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g) on crude mucin and sialic acid excretion in broiler chickens, at d 24 post hatching.

| Item                      | 0    | 8,000 | SEM | 0    | 15,000 | SEM | 0    | 8,000 | P-value | P × TI |
|---------------------------|------|-------|-----|------|--------|-----|------|-------|---------|--------|
| CM, g/kg excreta          | 317.2| 342.3 | 312.2| 353.1| 25.56  | 329.8| 332.7| 314.7| 347.7   | 0.911  |
| CM, g/kg DMI              | 70.2 | 71.8  | 57.5 | 77.4  | 4.97   | 71.0 | 67.4 | 63.8 | 74.6    | 0.479  |
| SA, mg/kg excreta         | 187.8| 270.0 | 189.1| 241.3| 23.73  | 228.9| 215.2| 188.4| 255.7   | 0.568  |
| SA, mg/kg DMI             | 40.2 | 50.7  | 34.1 | 48.5  | 5.56   | 45.3 | 41.3 | 37.1 | 49.6    | 0.477  |

1Abbreviations: CM, crude mucin; P, protease; SA, sialic acid; TI, trypsin inhibitor.

### Table 7. Effect of exogenous protease (PROT/kg) and purified trypsin inhibitor (TIU/g) on pancreas weight and proteolytic enzyme secretion in broiler chickens, at d 24 post hatching.

| Item                      | 0    | 8,000 | SEM | 0    | 15,000 | SEM | 0    | 8,000 | P-value | P × TI |
|---------------------------|------|-------|-----|------|--------|-----|------|-------|---------|--------|
| Pancreas wt., g           | 1.39 | 1.47  | 1.35| 1.32 | 0.075  | 1.42| 1.33 | 1.36 | 1.39    | 0.231  |
| Pancreas wt., g/kg BW     | 2.55 | 2.66  | 2.57| 2.50 | 0.143  | 2.60| 2.54 | 2.56 | 2.58    | 0.652  |
| Trypsin, U/mg             | 5.78 | 5.89  | 5.11| 4.62 | 0.247  | 5.83| 4.86 | 5.44 | 5.26    | < 0.001|
| Chymotrypsin, U/mg        | 4.53 | 4.04  | 5.25| 5.34 | 0.240  | 4.28| 5.30 | 4.88 | 4.69    | < 0.001|

1Abbreviations: P, protease; TI, trypsin inhibitor.
impact of TI on EAA secretion was higher for indispensable AA, which may have great nutritional consequences in birds. Interestingly, pancreatic enzyme secretion or pancreas weight was not affected by TI, indicative of no TI-induced hypertrophy. This is partly expected due to a lack of dietary protein needed for constant stimulation of the pancreas through the inhibitory actions of TI on trypsin. This also suggests that the pancreatic juice may not be a significant source for TI-induced EAA flow, however, this inference only applies to NFD conditions. Notwithstanding, digestive enzymes are degraded during the digestion process, absorbed into blood, reaccumulated by the pancreas, and reutilized (Rothman et al., 2002). Indeed, it is argued that a large fraction of the intact digestive enzymes secreted by the pancreas are absorbed and recycled in an enteropancreatic circulation, instead of being reduced to their constituent amino acids in the intestines (Rothman et al., 2002). Perhaps TI disrupts the digestive enzyme recycling process that may result in a surge in EAA flow, however, this need to be investigated.

Supplemental protease had no impact on the reduction or recovery of EAA secretion irrespective of dietary TI concentration, and contrary to a previous report (Huo et al., 1993) did not appear to be involved in inactivation or degradation of the TI. However, in vitro trials such as the report by Huo et al. (1993) may not be easily replicated in vivo. Perhaps fluctuations in the in vivo environment, especially in birds fed a protein-free non-physiological diet like the NFD, interferes with the exogenous protease ability to recognize and degrade the TI. However, these postulations have to be experimentally verified. Even more importantly, most commercially available proteases are chymotrypsin-like semi-alkaline endopeptidases and compared with an acid protease, may have limited capacity to degrade TI and other antigenic proteins (Hessing et al., 1996; Rooke et al., 1998). Although alkaline, the protease used in the current study may likely hydrolyze TI, but this reaction would more likely occur further down the gut (pH optima of Ronozyme ProAct is >6; Cowieson and Roos, 2016). However, this might be too late to prevent TI-induced EAA flow, especially if most of the secretions originated from the upper gastrointestinal tract (e.g., HCl, pepsin). Asides from lack of substrate with the use of NFD, the exogenous protease action may be limited by the low total EAA flow compared with a conventional diet. Furthermore, using data from Boisen and Verstegen (2000), which reported that the gross energy (GE) of amino acids ranges from 2,890 kcal/kg for Asp to 6,740 kcal/kg for Phe, we calculated the GE of the lost proteins based on their amino acid composition and gross flow. Similar to phytic acid (Cowieson et al., 2004), endogenous protein loss from the terminal ileum of broilers as a result of TI ingestion had considerable energetic consequences for the host. This is without considering the substantial amount of energy required in the synthesis of the endogenous proteins in the first place.

A major source of EAA secretion is the intestinal mucus glycoprotein. In the current study, and similar to phytic acid (Cowieson et al., 2004), TI increased the excretion of crude mucus and its metabolic marker, sialic acid (Jourdain et al., 1971) indicative of continuous sloughing of the mucus layer. However, contrary to previous reports (Peek et al., 2009; Cowieson and Roos, 2014) exogenous protease did not alleviate loss of mucoproteins from the gut. Cowieson and Roos (2014) showed a significant correlation between the AA profile of intestinal mucin and the effect of exogenous protease on AA digestibility and submits that part of the beneficial effect of exogenous protease may be mediated via a reduction in the loss of mucoprotein from the intestine. Although the mechanism by which this may occur is not clear, it was suggested (Cowieson and Roos, 2014) that exogenous protease may likely reduce the secretion of HCl and pepsin in the gastric phase of digestion, reducing the need for mucus as a protective agent in the intestine. However, as previously discussed, the observed lack of mucoprotective capacity by protease may be related to the nonphysiological nature of the NFD or the absence of protein in the feed or both. Intestinal mucoprotein is highly resistant to enzymatic hydrolysis (Montagne et al., 2004), compared to pancreatic secretions, which hinders AA recycling and is predominated by Thr, Pro, Ser, and Glu in the ileal flow (Lien et al., 2001; Ravindran, 2021). This disruption in thickness and fluidity of the mucosal layer by TI would also disrupt nutrient digestion, absorption and intestinal barrier function (Smirnov et al., 2004). Interestingly, a previous study (Sambeth et al., 1967) identified TI as a stimulant of gall bladder contraction in birds. The resultant increase in bile production will increase EAA flow, especially for taurine (data not included), which almost exclusively conjugates with bile acid in birds (Bremmer, 1958; Ravindran, 2021).

The current study also provides novel data on mineral digestibility in broiler chickens fed an experimental NFD and offers valuable insight into possible interactions of macro and trace minerals with TI. Feeding birds with NFD resulted in negative digestibility values for Fe, Mg, Cu, and K indicating increased endogenous flow at the terminal ileum, which was further exacerbated by added TI. Increased endogenous mineral excretion may be explained by the interactions of various cations with the mucus components, where divalent ions have a greater affinity than monovalent ions for the mucins (Powell et al., 1999). Similarly, Cowieson et al. (2004) and Woyengo et al. (2009) previously reported increased endogenous excretion of Fe, Mg, Na, and S in broiler chickens and pigs due to phytic acid ingestion. Phytic acid is a potent chelator of mineral ions (Maenz et al., 1999) and the observations in the current study suggests similar propensity of TI to form insoluble complexes with minerals, which is eventually excreted. Exogenous protease improved the digestibility of Cu, irrespective of dietary TI concentration in the current study. This is an interesting observation because aside from the primary function in enzyme systems within cells, Cu intake is the main determinant of bone strength (Roughhead and Lukaski, 2003). This is ascribed to a Cu-containing...
enzyme, lysyl oxidase, which is responsible for enhancing bone strength (Ilich and Kerstetter, 2000). In fact, Cu and Fe deficiency inhibits bone growth and decreases bone strength even when Ca and P levels are adequate (Medeiros et al., 1997). With few exceptions, the negative digestibility values observed in the current study suggest that the impact of protease may be more related to reductions in endogenous flow rather than improved dietary retention of the minerals. This may be a more appropriate description of the exogenous protease effect because of the consistent weight loss of birds as a result of being fed a NFD. It is unclear why addition of exogenous protease affected the digestibility of Zn and Mg in birds fed NFD containing TI. It is pertinent to state that a careful interpretation of these data may be required because mineral intake through the water source was unaccounted for in the current study. However, drinking water may not be a major source of minerals for broilers and due to high variability of individual daily water consumption, it is impossible to calculate mineral intakes from drinking water (Underwood, 1999). More importantly, complex interactions among minerals exist in the gut, which is a major cause of variation in availability and utilization or endogenous secretion. For instance, Zn absorption is impaired by Fe (Solomons and Jacob, 1981), while excess Zn interferes with Fe incorporation into ferritin (Settlemire and Matrone, 1967).

It can be concluded that the ingestion of TI by broilers increased the excretion of EAA, minerals, crude mucin, and sialic acid. Although supplementing with protease differentially affected proteolytic enzyme secretion by the pancreas, it did not reduce EAA losses, irrespective of added purified dietary TI. However, there were significant interactions between exogenous protease and TI for the AID of Zn, Mg, Cu, and Na. In particular, the AID of Cu and Mg increased with protease supplementation but protease reduced or caused no change in other mineral utilization in diets containing added TI. It should be noted that the effects observed in the current study were obtained in birds fed NFD, and it is unclear how these will be mediated in birds fed on conventional diets, containing SBM with variable TI concentrations. However, these results emphasize the impact of trypsin inhibitor on endogenous nutrient loss and hence should be considered when formulating poultry diets.

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DISCLOSURES

The authors declare that there is no conflict of interest.

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