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Optimizing the zymogram of exogenous proteases in broiler diets by in vitro simulated gastrointestinal digestion and response surface methodology

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Low protein digestibility causes intestinal flora imbalance, wet litter, and nitrogen pollution. The present study aimed to explore the optimal zymogram of exogenous acid protease (ACP), neutral protease (NEP), alkaline protease (ALP), and keratinase (KEA) in corn-soybean based diets for broilers. The hydrolysis performances of the four monocomponent proteases were presented by enzymatic hydrolysate gross energy (EHGE) and improved dry matter digestibility (IDMD), which were tested via the in vitro simulated digestion method. The optimal combination of the four proteases was predicted by the response surface method. Results showed that the optimal zymogram for 1 to 3-wk-old broiler diet was 1.80 U/g ACP, 2.30 U/g NEP, 29.30 U/g ALP, and 2.80 U/g KEA, and the EHGE and IDMD reached 94.65 Cal/g and 2.54%. The optimal zymogram for 4 to 6-wk-old broiler diet was 1.50 U/g ACP, 1.90 U/g NEP, 31.53 U/g ALP, and 3.10 U/g KEA, and the EHGE and IDMD reached 92.29 Cal/g and 2.47%. The performances of the predicted optimal zymogram were further verified by in vitro simulated digestion method. Collectively, the combined use of four proteases could improve the protein digestibility in broiler diets, which had better effect than monocomponent protease.

Keywords In vitro simulated digestion, Poultry diet, Protease, Response surface methodology, Zymogram

Dietary protein from animal and plant protein meals provides most of the amino acids for poultry. After swallowed, protein is catalyzed by proteases and digested into amino acids, which are then absorbed from gut lumen and transported across enterocytes into the portal circulation[1]. Factors such as protein hydrolyzation rate, amino acid releasing rate, and amino acid absorption rate are critical to the digestion of dietary protein in poultry[2]. Multiple proteases are involved in protein hydrolyzation, such as pepsin, trypsin, chymotrypsin, elastase, carboxypeptidase, and collagenase, and their concentrations and activities directly influence the level of hydrolysis[3]. Proteins that escape gastrointestinal digestion are prone to flow into the hindgut, which causes the problem of wet litter in birds[4]. Moreover, the microbial fermentation of undigested protein can lead to the formation of undesirable compounds such as ammonia, amines and indoles, which diminish the bird health and cause nitrogen pollution[2]. Therefore, it is widely accepted by poultry farmers using low-protein diet formulation, which has less intact protein-bound amino acids, more crystalline unbound amino acids, and more starch in diet compared to the standard diet[5]. Recent studies proved that a moderate reduction in dietary crude protein concentration could increase the ileal amino acid digestibility[6,7], and maintain the performance and processing yield of broilers[6,8].

Another strategy to improve the protein digestion in poultry is dietary supplement of exogenous proteases. Exogenous proteases can cleave proteinaceous anti-nutrients such as trypsin inhibitors, which leads to the enhancements of amino acid bioavailability and poultry growth performance[9]. Additionally, exogenous proteases can hydrolyze the peptide bonds. Based on reaction type, proteases can be categorized into exopeptidases which

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merely hydrolyze on the nitrogen or carbon terminal points of polypeptide, and endopeptidases which cleave non-terminal point in amino acids[10]. Currently, two-third of industrially essential proteases are manufactured by microbes, and the optimal pH conditions for the microorganism, as well as the microbial proteases range from 3.4 (aspartic protease synthesized by *Rhizopus oryzae*) to 11 (cellulase free thermostable protease synthesized by *Bacillus species JB-99*)[10]. Therefore, exogenous proteases can be classified as acid proteases, neutral proteases, and alkaline proteases as well. It is suggested that the use of multiple proteases combination can provide more better protein hydrolysis effect than the use of a single enzyme[11]. Considering the dynamic pH environment in the gut, dietary supplement of proteases combination was preferred in poultry farming, but the zymograms and effects varied[12,13].

Optimizing the combination of exogenous enzymes has always been challenging because it requires huge number of experimental animals and long-lasting time. In vitro assessment using simulated gastrointestinal tract model provides a simple, fast, and economic approach to predict the efficacy of different enzyme combinations on nutrients digestion, which has been used and proved solid in poultry[14,15]. Thus, in the present study, the optimal doses of four exogenous proteases are predicted using in vitro method, and their efficacies of the protein digestion in a corn-soybean meal basal diet of broiler were also evaluated.

Results

Hydrolysis performance and optimization of exogenous monocomponent protease on broiler diets

The hydrolysis performances of monocomponent protease on diet for broilers (1-wk-old to 3-wk-old) were tested via in vitro simulated gastrointestinal digestion system. The EHGE and IDMD were chosen as the parameters for the hydrolysis performance. As demonstrated in Fig. 1, the levels of supplemented monocomponent proteases

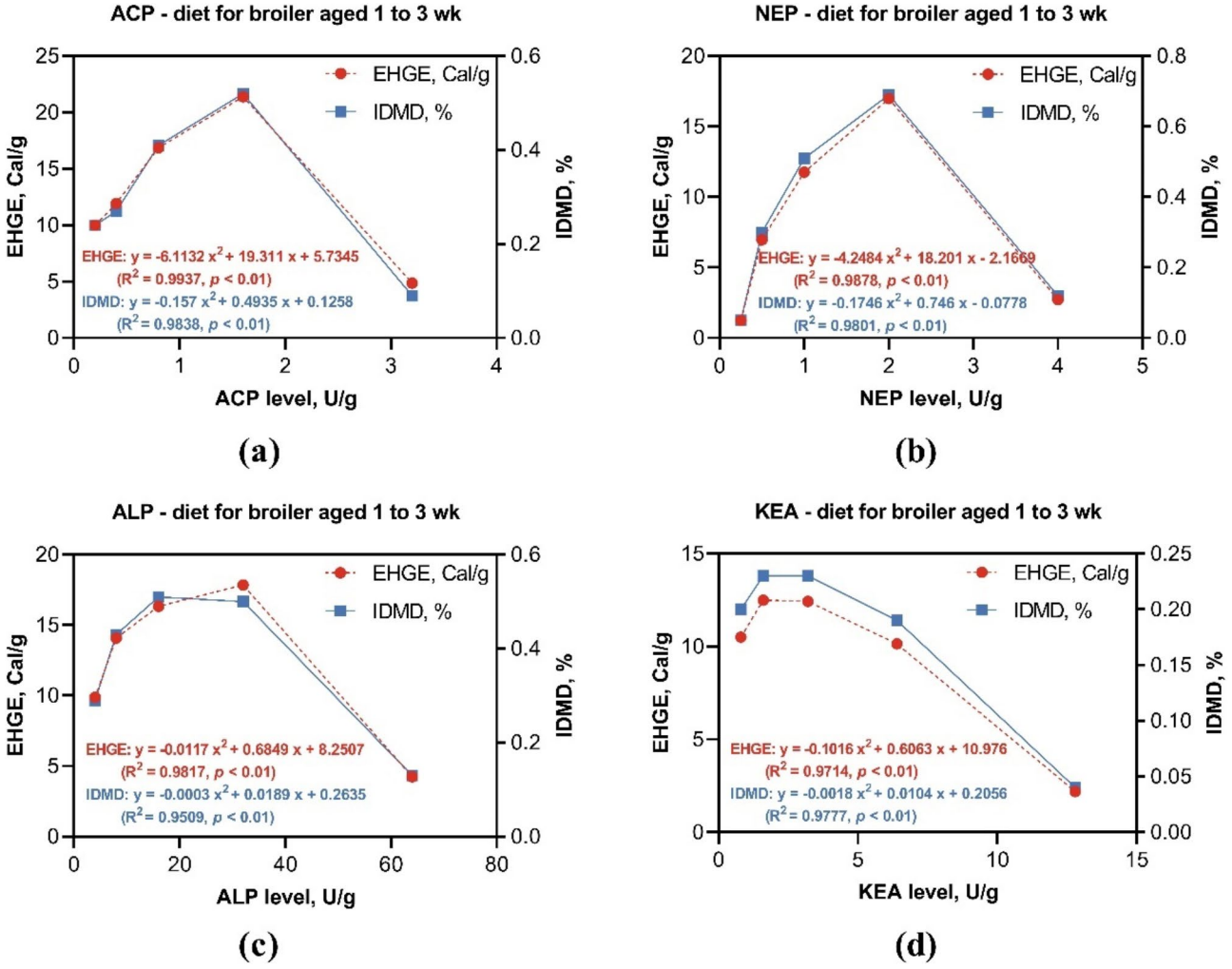

Fig. 1. Hydrolysis performances of exogenous monocomponent proteases on diet for broilers aged 1 to 3 wk, determined by the enzymatic hydrolysate gross energy (EHGE) and the improved dry matter digestibility (IDMD). (**a**) Acid protease (ACP); (**b**) neutral protease (NEP); (**c**) alkaline protease (ALP); (**d**) keratinase (KEA). The polylines indicate the variability of each indicator, that red scatter plots display the EHGE trend, and the blue solid lines show the IDMD trend. The data are demonstrated as means of 5 replicates.

all showed significant quadratic relationships with both EHGE and IDMD ($P<0.01$, $R^2>0.95$). Specifically, supplement of 1.58 U/g ACP in the diet provided the maximum EHGE at 20.98 Cal/g and the maximum IDMD at 0.51% (Fig. 1a). Supplement of 2.14 U/g NEP in the diet provided the maximum EHGE at 17.33 Cal/g and the maximum IDMD at 0.27% (Fig. 1b). Supplement of 29.27 and 31.50 U/g ALP in the diet provided the maximum EHGE at 18.27 Cal/g and the maximum IDMD at 0.56%, respectively (Fig. 1c). Supplement of 2.98 and 2.89 U/g ALP in the diet provided the maximum EHGE at 11.88 Cal/g and the maximum IDMD at 0.22%, respectively (Fig. 1d).

The same procedure was carried out on the diet for broilers aged 4 to 6 wk. As showed in Fig. 2, significant quadratic relationships were found between the supplemented monocomponent protease level and the EHGE ($P<0.01$, $R^2>0.97$), as well as the IDMD ($P<0.01$, $R^2>0.95$). In detail, supplement of 1.73 U/g ACP in the diet provided the maximum EHGE at 21.95 Cal/g and the maximum IDMD at 0.62% (Fig. 2a). Supplement of 2.12 U/g and 2.06 U/g NEP in the diet provided the maximum EHGE at 20.83 Cal/g and the maximum IDMD at 0.63%, respectively (Fig. 2b). Supplement of 30.48 and 33.83 U/g ALP in the diet provided the maximum EHGE at 26.11 Cal/g and the maximum IDMD at 0.55%, respectively (Fig. 2c). Supplement of 3.09 and 2.94 U/g ALP in the diet provided the maximum EHGE at 10.90 Cal/g and the maximum IDMD at 0.20%, respectively (Fig. 2d).

Response surface analysis of EHGE and IDMD on broiler diets supplement with exogenous proteases combination

The Box-Behnken designs of the 4 exogenous proteases and the predicted values of EHGE and IDMD on diets for broilers aged 1 to 3 wk and 4 to 6 wk were showed in Table 1. For the 1 to 3-wk-old broiler diet, the zymogram of 1.80 U/g ACP, 2.30 U/g NEP, 29.30 U/g ALP, and 2.80 U/g KEA performed the maximum IDMD and EHGE, which were 2.54% and 94.65 Cal/g respectively. For the 4 to 6-wk-old broiler diet, the zymogram of 1.50 U/g

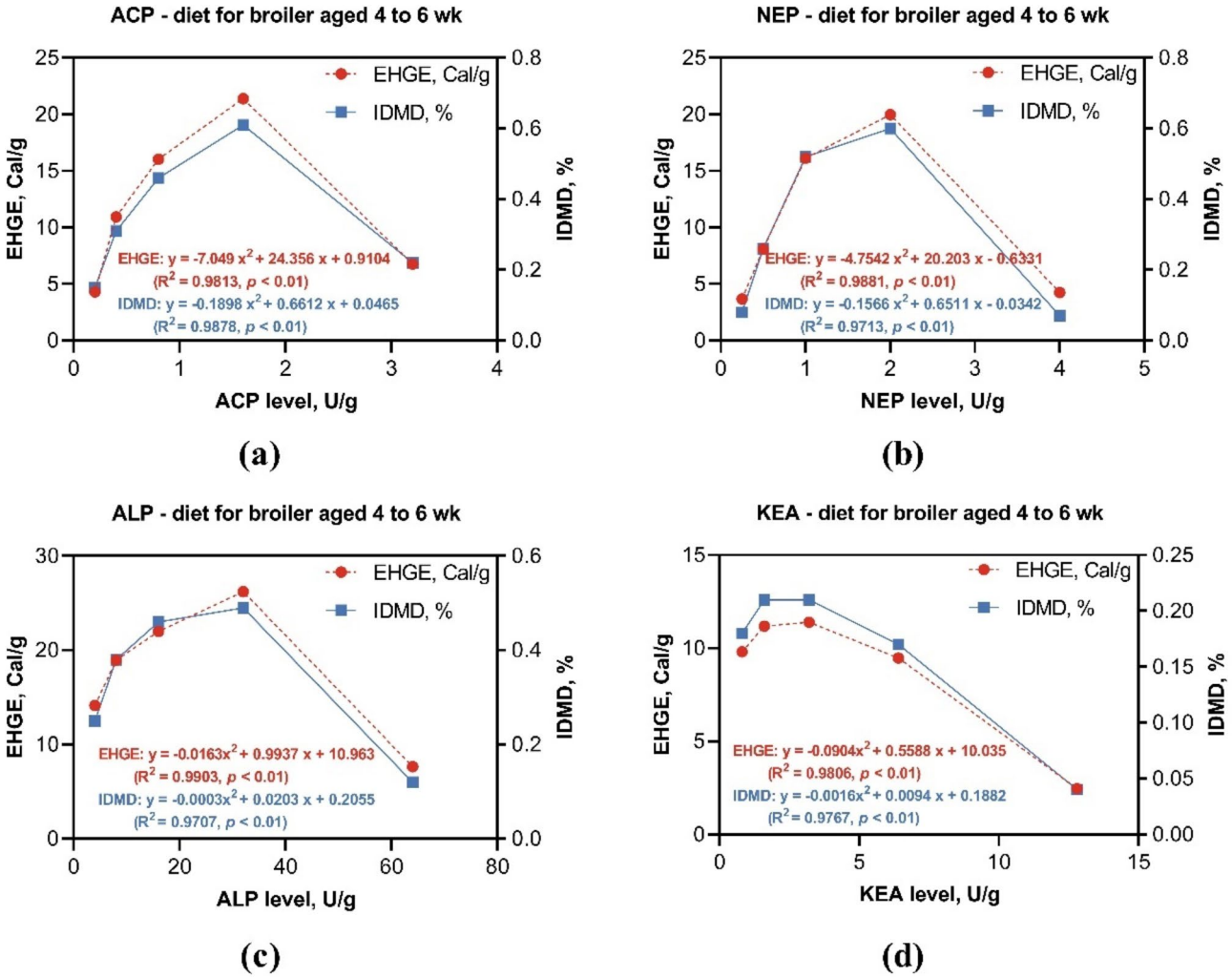

Fig. 2. Hydrolysis performances of exogenous monocomponent proteases on diet for broilers aged 4 to 6 wk, determined by the enzymatic hydrolysate gross energy (EHGE) and the improved dry matter digestibility (IDMD). (**a**) Acid protease (ACP); (**b**) neutral protease (NEP); (**c**) alkaline protease (ALP); (**d**) keratinase (KEA). The polylines indicate the variability of each indicator, that red scatter plots display the EHGE trend, and the blue solid lines show the IDMD trend. The data are demonstrated as means of 5 replicates.

Diet for 1 to 3 wk-old broiler						Diet for 4 to 6 wk-old broiler					
Supplement level, U/g				Improved dry matter digestibility, %	Enzymatic hydrolysate gross energy, Cal/g	Supplement level, U/g				Improved dry matter digestibility, %	Enzymatic hydrolysate gross energy, Cal/g
X_1 (Acid protease)	X_2 (Neutral protease)	X_3 (Alkaline protease)	X_4 (Keratinase)	y_1	y_2	X_1 (Acid protease)	X_2 (Neutral protease)	X_3 (Alkaline protease)	X_4 (Keratinase)	y_1	y_2
1.60	2.10	30.40	2.90	2.39	89.48	2.00	2.10	33.80	3.00	2.42	90.46
1.80	2.30	30.40	2.90	2.39	89.48	1.75	2.30	30.40	3.00	2.37	88.62
1.40	1.90	30.40	2.90	2.38	88.91	1.75	2.10	30.40	2.90	2.38	88.95
1.60	2.10	30.40	2.90	2.40	89.58	1.75	1.90	33.80	3.00	2.42	90.25
1.60	2.10	30.40	2.90	2.37	88.59	1.75	2.10	30.40	3.10	2.39	89.45
1.80	2.10	30.40	2.80	2.42	90.33	1.75	1.90	31.53	3.00	2.43	90.67
1.60	2.10	29.30	2.80	2.40	89.77	1.50	2.10	31.53	2.90	2.40	89.91
1.60	1.90	29.30	2.90	2.41	90.19	2.00	1.90	31.53	3.00	2.40	89.71
1.60	2.30	30.40	3.00	2.40	89.65	1.75	2.30	31.53	2.90	2.43	90.65
1.60	1.90	31.50	2.90	2.41	90.10	1.75	2.10	33.80	3.10	2.41	90.18
1.60	1.90	30.40	3.00	2.43	90.88	1.50	2.10	30.40	3.00	2.41	89.99
1.40	2.10	29.30	2.90	2.39	89.18	1.75	2.30	31.53	3.10	2.39	89.41
1.60	2.10	31.50	3.00	2.39	89.48	2.00	2.10	31.53	3.10	2.38	89.13
1.60	2.10	31.50	2.80	2.43	90.83	1.75	2.30	33.80	3.00	2.42	90.47
1.80	2.30	29.30	2.80	2.54	94.65	1.75	2.10	31.53	3.00	2.43	90.67
1.60	2.30	31.50	2.90	2.39	89.48	1.75	1.90	30.40	3.00	2.40	89.63
1.60	2.10	30.40	2.90	2.39	89.16	1.75	2.10	31.53	3.00	2.43	90.67
1.40	2.10	31.50	2.90	2.44	91.28	1.50	2.10	31.53	3.00	2.43	90.67
1.60	2.30	30.40	2.80	2.44	91.31	1.50	1.90	31.53	3.10	2.47	92.29
1.60	2.10	29.30	3.00	2.42	90.46	2.00	2.30	31.53	3.00	2.40	89.87
1.60	2.30	29.30	2.90	2.39	89.48	1.50	2.30	31.53	3.00	2.41	90.08
1.40	2.10	30.40	2.80	2.40	89.56	1.75	2.30	31.53	3.00	2.43	90.67
1.80	1.90	30.40	2.90	2.43	90.92	2.00	2.10	31.53	2.90	2.42	90.63
1.80	2.10	29.30	2.90	2.34	87.57	2.00	2.10	30.40	3.00	2.36	88.30
1.40	2.30	30.40	2.90	2.46	91.96	1.75	1.90	31.53	3.10	2.44	91.05
1.80	2.10	31.50	2.90	2.43	90.96	1.50	2.10	33.80	3.00	2.41	90.32
1.60	2.10	30.40	2.90	2.42	90.34	1.50	1.90	31.53	3.00	2.44	91.05
1.40	2.10	30.40	3.00	2.45	91.45	1.75	2.10	33.80	2.90	2.43	90.71
1.80	2.10	30.40	3.00	2.41	89.98	1.75	1.90	31.53	2.90	2.41	89.83

Table 1. Box-Behnken design of exogenous proteases with coded values and predicted values of IDMD and EHGE on diets for 1 to 3-wk-old and 4 to 6-wk-old broiler.

ACP, 1.90 U/g NEP, 31.53 U/g ALP, and 3.10 U/g KEA performed the maximum IDMD and EHGE, which were 2.47% and 92.29 Cal/g respectively.

Tables 2 and 3 presented the ANOVA analysis of the quadratic polynomial model for the optimization of the exogenous protease combinations on diets for broilers aged 1 to 3 wk and 4 to 6 wk, respectively. The quadratic polynomial model was applicable to the evaluations of exogenous proteases performance ($p_{model} < 0.001$), and the IDMD ($p_{Lack\ of\ fit} = 0.115$ and 0.247) and EHGE ($p_{Lack\ of\ fit} = 0.295$ and 0.062) were proper parameters representing the hydrolysis capabilities of protease combinations on diets for broilers aged 1 to 3 wk and 4 to 6 wk. It was noticeable that except for the interactions between ACP and ANP ($P = 0.067$), and NEP and ALP ($P = 0.724$) on the IDMD of 1 to 3-wk-old broiler diet, all four proteases and their interactions had significant influences on the IDMD and EHGE of broiler diets ($P < 0.05$).

The significant interactions between the tested proteases and the response metrics in broilers diets were demonstrated by three dimensional images. As demonstrated in Table 2, the effects of interactions between ACP and ALP ($P < 0.01$), ACP and KEA ($P = 0.003$), NEP and KEA ($P < 0.001$), and ALP and KEA ($P = 0.003$) on IDMD were significant in diet for broilers aged 1 to 3 wk, which were showed in Fig. 3a–d. And the effects of the interactions between all tested proteases on EHGE were significant in diet for broilers aged 1 to 3 wk ($P < 0.001$), which were showed in Fig. 3e–j. Referring to Table 3, the effects of the interactions between all tested proteases on IDMD and EHGE were significant in diet for broilers aged 4 to 6 wk ($P \leq 0.001$), which were showed in Fig. 4a–l.

Variables	y_1 (Improved dry matter digestibility)					y_2 (Enzymatic hydrolysate gross energy)				
	Sum of squares	Degrees of freedom	Mean square	*F* value	*p* value	Sum of squares	Degrees of freedom	Mean square	*F* value	*p* value
Model	0.024	14	0.002	225.25	< 0.001	34.110	14	2.440	320,000.00	< 0.001
X_1	0.002	1	0.002	274.24	< 0.001	2.780	1	2.780	364,000.00	< 0.001
X_2	< 0.001	1	< 0.001	22.95	< 0.001	0.260	1	0.260	34,228.66	< 0.001
X_3	0.008	1	0.008	1029.05	< 0.001	12.970	1	12.970	1,700,000.00	< 0.001
X_4	0.001	1	0.001	158.82	< 0.001	1.550	1	1.550	204,000.00	< 0.001
X_1X_2	< 0.001	1	< 0.001	3.94	0.067	0.046	1	0.046	6032.25	< 0.001
X_1X_3	0.0001	1	0.001	81.36	< 0.001	0.560	1	0.560	73,747.46	< 0.001
X_1X_4	< 0.001	1	< 0.001	13.02	0.003	0.150	1	0.150	19,941.31	< 0.001
X_2X_3	< 0.001	1	< 0.001	0.13	0.724	0.001	1	0.001	66.37	< 0.001
X_2X_4	0.005	1	0.005	628.76	< 0.001	6.720	1	6.720	881,000.00	< 0.001
X_3X_4	< 0.001	1	< 0.001	13.02	0.003	0.100	1	0.100	13,425.32	< 0.001
X_1^2	0.004	1	0.004	507.86	< 0.001	4.450	1	4.450	584,000.00	< 0.001
X_2^2	< 0.001	1	< 0.001	12.52	0.003	0.300	1	0.300	38,933.76	< 0.001
X_3^2	0.003	1	0.003	452.51	< 0.001	4.300	1	4.300	563,000.00	< 0.001
X_4^2	< 0.001	1	< 0.001	15.43	0.002	0.072	1	0.072	9426.56	< 0.001
Residual	< 0.001	14	< 0.001			< 0.001	14	0.000		
Lack of fit	< 0.001	10	< 0.001	3.58	0.115	< 0.001	10	0.000	1.82	0.295
Pure error	< 0.001	4	< 0.001			< 0.001	4	0.000		
Corrected. total	0.024	28				34.110	28			
R^2	0.996					1.000				

Table 2. ANOVA analysis for the quadratic polynomial model based on y_1 (Improved dry matter digestibility) and y_2 (Enzymatic hydrolysate gross energy) on diets for broilers aged 1 to 3 wk.

Variables	y_1 (Improved dry matter digestibility)					y_2 (Enzymatic hydrolysate gross energy)				
	Sum of squares	Degrees of freedom	Mean square	*F* value	*p* value	Sum of squares	Degrees of freedom	Mean square	*F* value	*p* value
Model	0.013	14	0.001	157.68	< 0.001	15.520	14	1.110	85,997.37	< 0.001
X_1	0.001	1	0.001	238.78	< 0.001	1.790	1	1.790	139,000.00	< 0.001
X_2	0.001	1	0.001	120.69	< 0.001	0.490	1	0.490	37,922.60	< 0.001
X_3	0.003	1	0.003	559.88	< 0.001	4.630	1	4.630	359,000.00	< 0.001
X_4	< 0.001	1	< 0.001	5.26	0.038	< 0.001	1	< 0.001	33.51	< 0.001
X_1X_2	< 0.001	1	< 0.001	39.35	< 0.001	0.320	1	0.320	24,633.09	< 0.001
X_1X_3	0.001	1	0.001	168.07	< 0.001	0.840	1	0.840	65,091.16	< 0.001
X_1X_4	0.002	1	0.002	279.83	< 0.001	2.220	1	2.220	172,000.00	< 0.001
X_2X_3	< 0.001	1	< 0.001	27.33	0.001	0.380	1	0.380	29,198.48	< 0.001
X_2X_4	0.001	1	0.001	214.24	< 0.001	1.520	1	1.520	118,000.00	< 0.001
X_3X_4	< 0.001	1	< 0.001	42.02	< 0.001	0.260	1	0.260	20,455.59	< 0.001
X_1^2	0.001	1	0.001	162.9	< 0.001	0.350	1	0.350	27,119.27	< 0.001
X_2^2	< 0.001	1	< 0.001	49.54	< 0.001	0.440	1	0.440	33,859.50	< 0.001
X_3^2	0.002	1	0.002	419.65	< 0.001	2.910	1	2.910	226,000.00	< 0.001
X_4^2	< 0.001	1	< 0.001	65.67	< 0.001	0.200	1	0.200	15,348.92	< 0.001
Residual	< 0.001	14	< 0.001			< 0.001	14	< 0.001		
Lack of fit	< 0.001	10	< 0.001	2.1	0.247	< 0.001	10	< 0.001	5.24	0.062
Pure error	< 0.001	4	< 0.001			< 0.001	4	< 0.001		
Corrected total	0.013	28				15.520	28			
R^2	0.994					1.000				

Table 3. ANOVA analysis for the quadratic polynomial model based on y_1 (Improved dry matter digestibility) and y_2 (Enzymatic hydrolysate gross energy) on diets for broilers aged 4 to 6 wk.

Verification of the optimal zymogram by in vitro simulated digestion method

In order to verify the hydrolysis performances of the predicted zymograms, the in vitro stimulated digestion method was conducted to evaluate the actual IDMD and EHGE of broiler diets. Each combination was tested five times. For 1 to 3-wk-old broiler diet, the actual IDMD and EHGE for the optimal protease combination

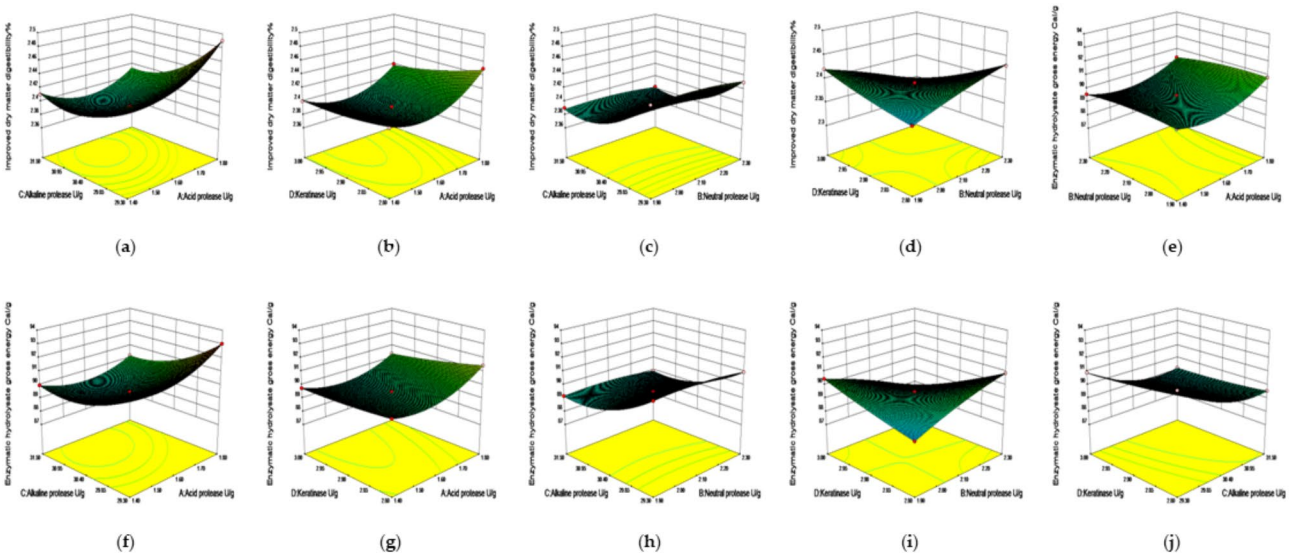

Fig. 3. Response surface and contour plots of the significant interactions between exogenous proteases on the improved dry matter digestibility (IDMD) and enzymatic hydrolysate gross energy (EHGE) on diet for broilers aged 1 to 3 wk. (**a**) to (**d**) represent the interactions of acid protease (ACP) and alkaline protease (ALP), ACP and keratinase (KEA), neutral protease (NEP) and ALP, NEP and KEA on the IDMD respectively. (**e**) to (**j**) represent the interactions of ACP and NEP, ACP and ALP, ACP and KEA, NEP and ALP, NEP and KEA, ALP and KEA on the EHGE respectively.

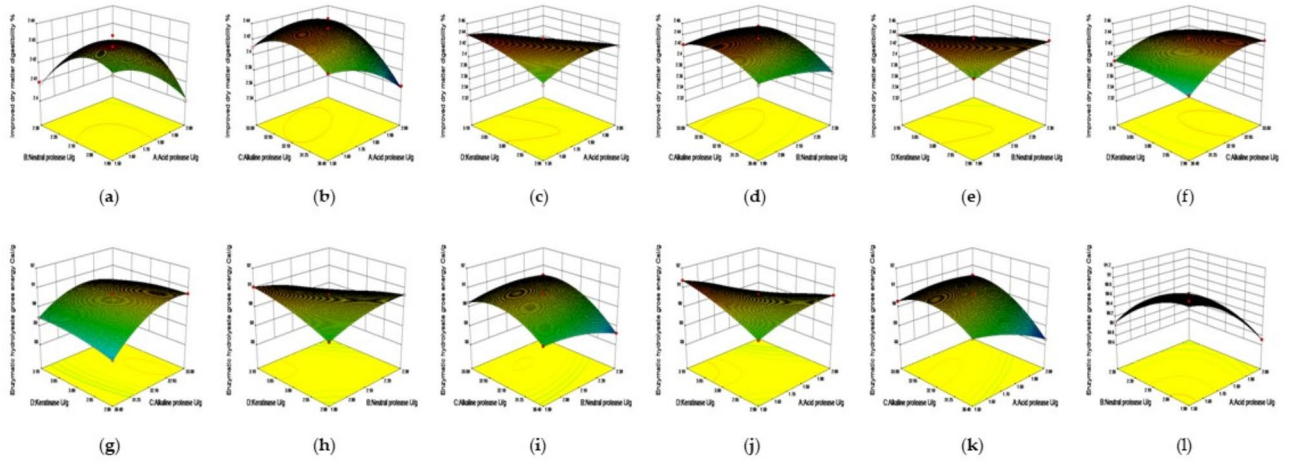

Fig. 4. Response surface and contour plots of the significant interactions between exogenous proteases on the improved dry matter digestibility (IDMD) and enzymatic hydrolysate gross energy (EHGE) on diet for broilers aged 4 to 6 wk. (**a**) to (**f**) represent the interactions of acid protease (ACP) and neutral protease (NEP), ACP and alkaline protease (ALP), ACP and keratinase (KEA), NEP and ALP, NEP and KEA, ALP and KEA on the IDMD respectively. (**g**) to (**l**) represent the interactions of ACP and NEP, ACP and ALP, ACP and KEA, NEP and ALP, NEP and KEA, ALP and KEA on the EHGE respectively.

(1.80 U/g ACP + 2.30 U/g NEP + 29.30 U/g ALP + 2.80 U/g KEA) were 2.51% and 92.06 Cal/g, which were only 1.18% and 2.74% lower than the predicted value (2.54% and 94.65 Cal/g). For 4 to 6-wk-old broiler diet, the actual IDMD and EHGE for the optimal protease combination (1.50 U/g ACP, 1.90 U/g NEP, 31.53 U/g ALP, and 3.10 U/g KEA) were 2.41% and 89.19 Cal/g, which were only 2.43% and 3.36% lower than the predicted value (2.47% and 92.29 Cal/g). Both results demonstrated that the predicted optimal zymogram of ACP, NEP, ALP, and KEA could effectively contribute to better hydrolysis performances on broiler diets.

Discussion

Protein digestibility is an important measure in evaluating the quality of protein source in animal feed. Excluding the differences in digestive capability of animal species, the protein digestibility is influenced by the portion of digestible proteins, the level of insoluble fiber, and the concentration of antinutritional factors[16]. For most

regions in the world, especially the developing countries where the protein digestibility and quality of feed are relatively low, dietary supplementation of exogenous protease is a common approach to stimulate the dietary protein digestibility[17,18]. It is also reported that exogenous protease can enhance the growth performance[19,20], strengthen the immune capability[21,22], modulate the intestinal microbial composition[23,24], and improve the qualities of animal products[25,26]. In vivo assays are considered the gold standard for assessing the nutrient digestion, which is time-consuming and requires the use of expensive isotope labeling of pure proteins and tracers[27]. While in vitro methods using simulated gastrointestinal tract provides a practical approach to evaluate nutrient digestibility, which has already been successfully used in ruminant species[28], pigs[29], and poultry[30,31]. Previous study by Ding et al.[32] agreed on the result that protease supplement in 1 to 3-wk-old broilers diet significantly improved the hydrolysate gross energy and dry matter, especially the crude protein digestibility, partially because of the benefit effects of protease on the endogenous trypsin activity and intestinal morphology. Meanwhile, significant quadratic relationships were found among the monocomponent proteases and protein digestibility related parameters. In our earlier study, monocomponent carbohydrase also showed quadratic relationships with reduced sugar release amount and improved dry matter digestibility[14]. It suggested that proper rate of exogenous proteases supplement in diet was required to achieve the improved protein digestion performance.

The effect of exogenous protease on the digestion of dietary protein is determined by factors including feed formulation[33], pH[34], animal species[35], and inclusion rate[36]. Due to the complexity in the gastrointestinal construction and physiological condition, the mixing uses of different exogenous enzymes were tested by researchers. Cho et al.[37] incorporated a multi-protease supplementation in amino acid-deficient broiler diets, and found improved feed efficiency and ileum digestibility compared to the group without protease supplementation. Similarly, the present study found that the four exogenous proteases individually or interactively increased the IDMD and EHGE of 1 to 3-wk-old and 4 to 6-wk-old broiler diets, which proved that mixed incorporation of the exogenous proteases could improve the protein hydrolysis and digestion in poultry diet. Furthermore, the optimal combinations of the 4 proteases and the associated IDMD and EHGE in poultry diets were predicted by the response-surface method, and verified by in vitro simulated gastrointestinal digestion method. Comparing the protein digestibility related parameters in diets supplemented with monocomponent protease and proteases combination, it could be found that proteases combinations had better effect on protein digestibility than monocomponent protease did. However, Zheng et al.[38] showed inconsistent result that diet supplemented with combination of proteases did not provide better protein digestibility in broilers comparing to monocomponent protease. There were several possible reasons for the situation. Firstly, the protein source in feed had anti-nutritional factors, which might influence the hydrolysis effect of exogenous protease[39]. Secondly, the effect of protease supplementation was related to the dosage[40]. High dosage exogenous protease supplementation might not promote the protein utilization, but inhibit the secretion of endogenous protease and jeopardize the hydrolyzation.

Materials and methods

The study was approved by Hunan Institute of Animal Husbandry and Veterinary Medicine (approval No. HIAHVM202209). All experiments were performed in accordance with relevant guidelines and regulations. We firstly used the in vitro simulated gastrointestinal model evaluating the effects and optimal usages of the monoproteases on the protein hydrolyzation of corn-soybean based diets for broilers. Secondly, we used the response surface method to optimize the protease combination for the best protein hydrolyzation for broiler diets.

A response surface method with a three-level, four-variable Box-Behnken design were used for the optimization of the protease combination.

Exogenous proteases and diets compositions

The exogenous proteases used in the present study included acid protease (ACP), neutral protease (NEP), alkaline protease (ALP), and keratinase (KEA), which were derived from *Aspergillus niger* (50,000 U/mL), *Bacillus subtilis* (50,000 U/mL), *Bacillus subtilis* (100,000 U/mL), and *Bacillus chlamydiae* (200,000 U/mL), respectively. These proteases were provided by Shandong Lonct Enzymes Co., Ltd. (Linyi, Shandong, China).

The compositions of corn-soybean based diets for broilers at 1 to 3 wk and 4 to 6 wk were based on arbor acres plus standard, which were showed in Table 4.

In vitro simulated gastrointestinal model

A third generation of simulated monogastric animal digestion system (SDS-III, Zhongben Intelligent Technology Development Co., Ltd., Changsha, China) was used for the in vitro digestion experiment. The operation of the system was based on Zhao's description[41]. Briefly, the simulated gastric phase buffer was composed of 5.18 g sodium chloride, 0.50 g potassium chloride, and 15.60 g sodium dihydrogen dihydrate dissolving in distilled water to 1000 mL, and the final pH at 41 °C was adjusted to 2.80. The simulated small intestine phase buffer was composed of 13.68 g anhydrous disodium hydrogen phosphate and 50.44 g sodium dihydrogen phosphate dihydrate dissolving in distilled water to 1000 mL, and the final pH at 41 °C was adjusted to 6.91. When testing, diet sample substrate was put in 50 mL sterile enzyme-free corning centrifuge tubes, added with 8 mL pepsin solution (0.27 g pepsin dissolved in 100 mL gastric buffer) and 1 mL chloramphenicol solution, followed with gentle shakes. One mL of the tested exogenous protease was added, and the mixture was kept in a water bath at 41 °C for 4 h. After that, 6 mL intestinal buffer and 1.6 mL pancreatin solution (1.87 g trypsin dissolved in 100 mL distilled water) were added to the tested tubes, followed with shaking in 41 °C water baths for 15 h. Finally, all samples were placed in an ice to terminate the enzyme reaction, then centrifugated at 5000×*g* for 10 min to

Items	1 to 3 wk	4 to 6 wk
Content, %		
Corn	53.68	60.21
Soybean meal (43%)	25.50	18.00
Wheat middling	5.00	5.00
Peanut meal (46%)	3.00	2.00
Cottonseed meal (46%)	2.00	3.00
Corn protein meal (60%)	2.00	2.00
Feather meal (80%)	2.00	3.00
Soybean oil	1.80	2.20
Limestone	1.68	1.48
CaHPO4	1.18	1.10
L-Thr (99%)	0.17	0.15
L-Lys (70%)	0.70	0.70
DL-Met (98%)	0.29	0.16
Premix[a]	1.00	1.00
Total	100.00	100.00
Nutrient levels[b], %		
Metabolic energy, MJ/kg	12.54	12.96
Crude protein	22.00	20.00
Ether extract	4.50	5.00
Lysine	1.17	1.01
Methionine	0.61	0.45
Threonine	0.81	0.72
Methionine + cystine	0.91	0.76
Calcium	1.05	0.94
Total phosphate	0.68	0.65
Available phosphate	0.47	0.45

Table 4. Compositions and nutrient levels of basal diets (air-dry basis) for broilers aged 1 to 3 wk and 4 to 6 wk. [a]The premix provided the following per kg of diets: VA 10,000.00 IU, VB1 5.60 mg, VB2 11.00 mg, VB6 8.00 mg, VB12 0.02 mg, VD3 3000.00 IU, VE 40.00 IU, VK3 2.50 mg, biotin 0.15 mg, folic acid 2.00 mg, D-pantothenic acid 32.00 mg, nicotinic acid 60.00 mg, antioxidant 100.00 mg, Cu (as copper sulfate) 10.00 mg, Fe (as ferrous sulfate) 80.00 mg, Mn (as manganese sulfate) 60.00 mg, Zn (as zinc sulfate) 35.00 mg, I (as potassium iodide) 0.42 mg, Se (as sodium selenite) 0.30 mg. [b]Nutrient levels were calculated values.

	Supplement level, U/g			
Treatment	Acid protease	Neutral protease	Alkaline protease	Keratinase
1	0.20	0.20	0.25	0.25
2	0.40	0.40	0.50	0.50
3	0.80	0.80	1.00	1.00
4	1.60	1.60	2.00	2.00
5	3.20	3.20	4.00	4.00

Table 5. Supplement levels of monocomponent protease.

retrieve the supernatants, which were stored at 4 °C. The undigested residue in tubes was washed with distilled water three times, dried at 65 °C overnight, and incubated at 105 °C for 4 h.

In vitro simulated digestion of diets supplemented with monocomponent protease

A single-factor complete random design method was used to evaluate the effects and optimal usages of the tested exogenous monocomponent protease in the in vitro digestion experiment. Each monocomponent protease was arranged with 5 supplement levels (Table 5), and each supplement level had 5 replicates.

Optimization of proteases combinations

A response surface method with a three-level, four-variable Box-Behnken design were used for the optimization of the protease combination. A total of 29 different test groups were designed, and the independent variables and levels were showed in Table 6.

Independent variable	Factor	Coded and actual levels, U/g		
		−1	0	1
Acid protease	x_1	1.40	1.60	1.80
Neutral protease	x_2	1.90	2.10	2.30
Alkaline protease	x_3	29.30	30.40	31.50
Keratinase	x_4	2.80	2.90	3.00

Table 6. Independent variables and their levels for zymogram optimization in the Box Behnken design.

$$\mathrm{EHGE\ (Cal/g)} = (\mathrm{GE_1} - \mathrm{GE_2})\,/\mathrm{M_1} \tag{1}$$

where GE_1 is the gross energy of the sample before digestion (Cal), GE_2 is the gross energy of the undigested sample after digestion (Cal), M_1 is the dry matter weight of the sample before digestion (g). Gross energy of samples are measured using a bomb calorimeter (Parr 6400 Calorimeter, Parr Instrument Company, Moline, USA).

$$\mathrm{IDMD\ (\%)} = (\mathrm{M_1} - \mathrm{M_2})\,/\mathrm{M_1} \times 100 \tag{2}$$

where M_1 is the dry matter weight of the sample before digestion (g), M_2 is the dry matter weight of the undigested sample after digestion (g).

Statistical analysis

Regression analysis was conducted in Statistical Package for the Social Science 19.0 (IBM, Armonk, USA). A probability of $P<0.05$ was considered statistically significant. Regarding to the response surface method, the experiment design, model calculation, and graph drawing were performed in Design Expert Software (Version 8.0.6, Stat-Ease Inc., Minneapolis, USA).

Conclusion

In conclusion, in the present study we predicted the optimal combination of 4 exogenous protease, including ACP, NEP, ALP, and KEA, supplementing in diets for broilers aged 1 to 3 wk and 4 to 6 wk, and calculated the IDMD and EHGE for the diets incorporated with the optimal zymogram. It provides solid support for the use of these exogenous proteases in broiler farming. However, further studies are keenly needed before the use of this formula, including but not limited to testing their effect in broilers of different breeds, and in broilers raised in different conditions.

Data availability

All data generated or analyzed during this study are included in this article.

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Author contributions

Conceptualization: Q.D.; methodology: Shengli Liu; software: Y.L.; formal analysis: Y.L.; investigation: Q.D.; resources: Shengli Liu; data curation: Shijie Liu; writing-original draft: Y.L. and Shijie Liu; writing-review and editing: F.C.; all authors have read and agreed with the final version of the manuscript.

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Declarations

Competing interests

Shengli Liu was employed by Shandong Lonct Enzymes Co. Ltd. during the time that the experiment was conducted and the paper was composed. All authors declared that no financial and personal relationships with other people or organizations could inappropriately influence the work. There was no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Additional information

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