Overexpression of heat shock protein 70 and its relationship to intestine under acute heat stress in broilers: 1. Intestinal structure and digestive function[1]

Y. Hao, X. H. Gu,[2] and X. L. Wang

State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, China

ABSTRACT The objective of this study was to investigate the relationship between heat shock protein 70 (*HSP70*) overexpression and intestinal structure and digestive function in heat-stressed broilers. In total, 240 male broilers were injected intraperitoneally with L-(1)-glutamine (0.75 mg/kg of BW) or quercetin (5 mg/kg of BW). Twenty-four hours later, they were heat-stressed for 0, 2, 3, 5, and 10 h, respectively, under 36 ± 1°C. The *HSP70* protein and mRNA expression were obviously elevated at 3 h of heat stress, and glutamine induced the overexpression of *HSP70* in the jejunal mucosa at different heat-stress times ($P < 0.01$). No significant change of jejunal villus height, crypt, and villus height:crypt ratio were observed after heat stress, and there were no effects of *HSP70* overexpression on intestinal morphology under heat stress. The overexpression of *HSP70* significantly increased alkaline phosphatase activity at 3 h of heat stress ($P < 0.01$). There was a strong correlation between *HSP70* expression and the digestive enzyme activity ($P \leq 0.001$). The overexpression of *HSP70* significantly increased the amylase, lipase, and trypsin activity under heat stress ($P < 0.001$). These results demonstrated that glutamine was a good *HSP70* enhancer to establish an *HSP70* overexpression model. Although the overexpression of *HSP70* did not change intestinal morphology conditions, it significantly increased broiler digestive enzyme activity under heat stress.

Key words: heat shock protein 70, broiler, intestine, digestive enzyme, alkaline phosphatase

2012 Poultry Science 91:781–789
http://dx.doi.org/10.3382/ps.2011-01627

INTRODUCTION

Heat stress is one of the most challenging environmental conditions affecting commercial poultry, and it causes the loss of revenue that ranges into millions of dollars each year (Mahmoud et al., 2003). Exposure of chickens to heat stress causes significant behavioral and physiological responses (Altan et al., 2003). Thermal stress exerts negative influence on feed intake, BW gain (Geraert et al., 1996), neuroendocrine, and immune function as well as on mortality rates (Smith, 1993). Heat stress seriously harms animal welfare and productivity, and therefore, becomes one of the major concerns for the poultry industry, especially in the hot regions of the world.

When living organisms are exposed to thermal stresses, the synthesis of most proteins is delayed, but a group of highly conserved proteins known as heat shock proteins or heat stress proteins (**HSP**) is rapidly synthesized (Al-Aqil and Zulkifli, 2009). These HSP play an important role in the survival of stressed cells and the stabilization of the internal environment (Gabai et al., 1997).

According to the homology and molecular weights, HSP can be classified into 3 main families: HSP90 (~85–90 kDa), HSP70 (~68–73 kDa), and low molecular weight HSP (~16–47 kDa; Basu et al., 2002). Among the HSP, HSP70 is one of the most conserved and important protein families and has been studied extensively (Deane and Woo, 2005; Ming et al., 2010).

It is well known that the intestine plays an important role in terms of being a body barrier, for digestion, and for absorption of nutrients and immunity, hence it becomes the target organ in stress response. In recent years, studies have found that HSP70 is induced in the gastric mucosal cells and epithelial cells of the jejunum and ileum, which are exposed to a variety of stress factors. Studies of the protective effects of HSP70 on the intestine mainly focus on rat, mice, or intestinal epithelial cells in vitro (Ehrenfried et al., 1995; Wischmeyer et al., 1997; Ren et al., 2001; Ohkawara et

Received May 25, 2011.
Accepted September 24, 2011.
[1]This investigation was financially supported by the National Natural Science Foundation of China (NSFC 30871797).
[2]Corresponding author: guxianhong@vip.sina.com

al., 2006). Although heat stress can induce intestinal injury in broiler chickens (Quinteiro-Filho et al., 2010), to the best of our knowledge, there is no information on the relationship between HSP70 activity and intestinal structure and digestive function in poultry under stressful conditions.

The most important nutrient for the gut, L-(1)-glutamine (**Gln**), is known to be protective of the intestinal epithelium and a potent enhancer of the synthesis of HSP both in vivo and in vitro (Wischmeyer et al., 1997; Kojima et al., 1998). Quercetin is a nonspecific chemical inhibitor of HSP, which inhibits not only HSP70 induction but also the cytoprotection provided by Gln supplementation (Wischmeyer et al., 1997). Thus, the present study was designed to use Gln and quercetine to evaluate the relationship between *HSP70* expression and intestinal structure and digestive function in the heat-stressed broiler.

MATERIALS AND METHODS

Experimental Birds

In total, 300 male broiler chicks (1 d old; Arbor Acres Poultry Breeding Co., Beijing, China) were housed in wire-floored cages in an environmentally controlled room with continuous light. The room temperature was maintained at 35°C when the chickens were at the age of 1 to 3 d old; afterward, the temperature was gradually reduced until 22°C. All birds were inoculated with an inactivated infectious bursal disease vaccine on d 14 and 21 and with a Newcastle disease vaccine on d 7 and 28. The birds had access to feed and water ad libitum. The diet was formulated to meet or slightly exceed the nutrient requirements suggested by the NRC (1994). All procedures were approved by the Animal Care and Welfare Committee of the Chinese Academy of Agricultural Sciences (Beijing, China).

Experimental Design

Two hundred forty broilers with similar BW were put into 24 cages (10 birds/cage) and randomly allocated to 4 treatments, including control, enhancer, inhibitor, and enhancer + inhibitor at 21 d of age. There were 6 cages per treatment. These birds were transported into a temperature-controled metabolic chamber. On 36 d of age, the birds were injected intraperitoneally with 0.5 mL of saline, Gln (0.75 mg/kg of BW), quercetin (5 mg/kg of BW), and Gln (0.75 mg/kg of BW) + quercetin (5 mg/kg of BW), respectively. After injection, they were put back into their original cages. Twenty-four hours later, all of the chickens in their original cages were moved to the environmental chamber set controlled at 36°C. They suffered from acute heat stress under 36 ± 1°C for stress times that were respectively 0, 2, 3, 5, and 10 h.

Sample Collection

During the 0, 2, 3, 5, and 10 h in the heat-stress condition, chickens that weighed similarly in each replicate per treatment average were selected and immediately killed by cervical dislocation. Longitudinal sections of jejunum tissue were obtained and flushed with 5 mL of saline. The tissues were then fixed in 3 to 5 volumes of 4% phosphate-buffered formaldehyde (pH 7.2). A homogeneous jejunal digesta sample was collected by massaging the tract from both ends. The digesta samples were stored immediately at −70°C until analysis. The mucosal samples of the jejunum were collected using glass slides and stored at −70°C until analysis.

Western Blot Analysis

Jejunal mucosa samples were vortexed for lysis for a few seconds every 15 min at 4°C for 1 h and centrifuged at 15,000 × *g* for 5 min at 4°C. The supernatants were assayed. The samples were heated at 95°C for 5 min and cooled on ice. Following the centrifugation at 15,000 × *g* for 5 min, 20 μL aliquots were resolved by electrophoresis (Bio-Rad, Richmond, CA) on 12% SDS-PAGE before being transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). After blocking with TBST (0.05% Tween 20, 100 m*M* Tris-HCl, and 150 m*M* NaCl, pH 7.5) containing 5% skim milk for 30 min at room temperature, the membranes were incubated with primary antibodies, anti-mouse HSP70 (Stressgen, Gentaur Bvba, Brussels, Belgium), in a dilution of 1:4000 at 4°C overnight. After washing in TBST 3 times, the membranes were incubated with a horseradish peroxidase-labeled secondary antibody (goat anti-mouse IgG, diluted in 1:10000; Beijing CoWin Biotech Co. Ltd., Beijing, China) for 40 min. The antibody-specific protein bands were visualized with an electrochemiluminescence substrate using a gel-imaging system (Tanon Science and Technology, Shanghai, China) with Image Analysis Software (National Institutes of Health, Bethesda, MD).

mRNA Expression Analysis

The HSP70 mRNA levels in mucosal samples of jejunum were analyzed by quantitative real-time reverse-transcription PCR (Tchernitchko et al., 2002). The total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. One microgram of the total RNA was reverse-transcribed to complementary DNA by using a SuperScript III Reverse Transcriptase kit (Invitrogen). Both HSP70 and β-actin were amplified in the ABI SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) from the complementary DNA with specific primers. The following quantitative real-time reverse-transcription PCR primers were used: β-actin (GenBank NM205518) forward 5′-AACCGGACTGTTACCAACACC-3′, reverse

5′-AGACTGCTGCTGACACCTTCAC-3′; and *HSP70* (GenBank NM001006685) forward 5′-CGTCAGTGCTGTGGACAAGAGTA-3′, reverse 5′-CCTATCTCTGTTGGCTTCATCCT-3′. Amplification was initiated by 2- and 5-min incubation periods at 50 and 95°C, respectively, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. Relative standard curves were obtained by plotting the cycle threshold (**Ct**) obtained after PCR amplification of serial dilutions of a steady quantity of a plasmid containing the corresponding complementary DNA. The PCR products of *HSP70* were normalized to the β-actin level.

Small Intestinal Morphology

Fixed intestinal samples were prepared by using conventional paraffin embedding techniques. Samples were sectioned at a 6-μm thickness and stained with hematoxylin-eosin. Villus height and crypt depth were measured according to Wu et al. (1996) under a light microscope (CK-40, Olympus Co., Tokyo, Japan). A total of 10 intact, well-oriented, crypt-villus units were selected in triplicate for each intestinal cross-section. Villus height was measured from the tip of the villus to the villus-crypt junction; crypt depth was defined as the depth of the invagination between adjacent villi. The histological analysis was performed by an investigator who was unaware of the origin of the tissue sections.

Alkaline Phosphatase and Digestive Enzyme Analyses

The small intestinal mucosal samples were diluted 10-fold, based on the sample weight with saline, to form homogenates. The samples were then centrifuged at 2,500 × *g* at 4°C for 10 min. The supernatants were determined according to the assay kit's instructions, obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). One unit of alkaline phosphatase activity was defined as the production of 1 mg of nitrophenol per gram of jejunal mucosal protein. Total protein concentration was determined using the Coomassie brilliant blue protein assay.

The concentrations of amylase, lipase, and trypsin activity in the jejunal digesta were determined by kits (Nanjing, China) as described by the manufacturer.

Statistical Analysis

All statistical analyses were performed using SAS software (version 8.2; SAS Institute, Cary, NC). Statistical analyses were performed using the one-way ANOVA to estimate the effects of treatment and time, respectively. Correlation analysis was performed by a linear regression test. Differences among means were tested using Duncan's multiple range tests. Means were considered significantly different for values of $P < 0.05$.

RESULTS

Levels of HSP70 in Jejunal Mucosa

The level of HSP70 protein in the jejunal mucosa of heat-stressed broilers is displayed in Table 1. No significant difference of HSP70 among the groups was found before heat stress. The level of HSP70 in the enhancer group was significantly higher than that in the control after 3 h of heat stress ($P < 0.001$). This indicates that using glutamine to establish an overexpression model of HSP70 is successful. However, the protein expression of *HSP70* in the inhibitor group was only significantly lower than that of the control after 3 h of heat stress ($P < 0.001$). There is a significant difference between expression levels of *HSP70* after 3 h of heat stress. The level of HSP70 in the enhancer group was significantly higher than that in the enhancer + inhibitor group after 3 h of heat stress ($P < 0.001$). Furthermore, there were significant effects from different heat-stress times on the expression of HSP70 ($P < 0.01$).

mRNA Expression in Jejunal Mucosa

Table 2 demonstrates the *HSP70* mRNA expression during heat stress. There was no significant difference in *HSP70* mRNA expression before heat stress. Compared with that of the control, *HSP70* mRNA expression was significantly upregulated in the jejunal mucosa

Table 1. Levels of heat shock protein 70 protein expression in the jejunal mucosa of broilers under heat stress[1]

Time (h)	Control	Enhancer	Inhibitor	Enhancer + inhibitor	SEM	*P*-value
0	0.70^{B}	0.75^{C}	0.68^{B}	0.71^{D}	0.019	0.1293
2	0.73^{B}	0.95^{AB}	0.88^{A}	0.93^{A}	0.055	0.0734
3	$0.93^{A,b}$	$1.11^{A,a}$	$0.70^{B,c}$	$0.76^{CD,c}$	0.026	<0.0001
5	$0.59^{C,b}$	$0.81^{BC,a}$	$0.68^{B,b}$	$0.85^{B,a}$	0.029	0.0009
10	$0.72^{B,c}$	$0.76^{C,b}$	$0.71^{B,c}$	$0.80^{BC,a}$	0.010	0.0007
SEM	0.020	0.005	0.018	0.019		
P-value	<0.0001	0.0036	<0.0001	0.0001		

[a–c]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

[A–D]Means in the same column with no common superscripts differ significantly ($P < 0.05$).

[1]n = 3 for each treatment group. The results are given as relative intensity normalized to β-actin (heat shock protein 70/β-actin).

Table 2. Levels of heat shock protein 70 mRNA expression in the jejunal mucosa of broilers under heat stress[1]

Time (h)	Control ($\times 10^{-3}$)	Enhancer ($\times 10^{-3}$)	Inhibitor ($\times 10^{-3}$)	Enhancer + inhibitor ($\times 10^{-3}$)	SEM ($\times 10^{-4}$)	*P*-value
0	2.01^{C}	2.63^{C}	1.74^{D}	1.33^{C}	3.41	0.1295
2	$2.23^{C,c}$	$6.78^{A,a}$	$4.48^{A,b}$	$2.36^{B,c}$	2.69	<0.0001
3	$3.41^{A,b}$	$5.22^{B,a}$	$3.31^{B,b}$	$3.90^{A,b}$	2.91	0.0058
5	$3.42^{A,b}$	$4.04^{B,a}$	$1.57^{D,d}$	$2.48^{B,c}$	1.81	<0.0001
10	$2.82^{B,b}$	$4.06^{B,a}$	$2.43^{C,b}$	$2.59^{B,b}$	1.31	<0.0001
SEM	0.14	0.40	0.18	0.22		
P-value	<0.0001	0.00030	<0.0001	0.0002		

[A–D]Means in the same column with no common superscripts differ significantly ($P < 0.05$).

[a–d]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

[1]n = 3 for each treatment group. The results are given as relative intensity normalized to β-actin (heat shock protein 70/β-actin).

of the enhancer group after heat exposure ($P < 0.01$). The mRNA expression of *HSP70* in the inhibitor group was only significantly lower than that of the control after 5 h of heat stress ($P < 0.001$). The amounts of *HSP70* mRNA in the enhancer were significantly higher than those in the inhibitor and the enhancer + inhibitor groups during heat stress ($P < 0.01$). A significant difference from heat-stress times on the expression of *HSP70* was also detected ($P < 0.01$).

Morphological Analysis

To examine the effects of HSP70 on small intestinal morphology under heat stress, jejunal sections stained with hematoxylin-eosin were examined by light microscope. No significant difference was observed in the villus height, crypt depth, or the villus height:crypt ratio in each group during the different heat-stress times. There was also no significant difference in villus height between groups during heat stress. No effect of HSP70 was found on crypt depth between the groups, except at 5 h of heat stress. For the villus height:crypt ratio, a significant difference between groups was observed only after 10 h of heat stress (Table 3).

Alkaline Phosphatase Activity

Figure 1 demonstrates the alkaline phosphatase activity in the jejunal mucosa of broilers under heat stress. Before heat stress, the alkaline phosphatase activity was significantly higher in the enhancer than in the other groups ($P < 0.001$). Early in the heat stress (2–3 h), there was no significant difference between groups. Then, with the increasing amount of time of heat stress (5–10 h), a significant difference in the alkaline phosphatase activity between groups was found ($P < 0.01$).

Table 3. Relationship between the overexpression of heat shock protein 70 and the intestinal morphology under heat stress[1]

Item	Control	Enhancer	Inhibitor	Enhancer + inhibitor	SEM	*P*-value
Villus height (μm)						
0 h	797.87	804.03	694.87	779.80	58.01	0.5473
2 h	918.27	989.60	950.84	883.37	69.32	0.7382
3 h	962.58	745.17	869.17	836.50	72.49	0.2791
5 h	753.57	794.37	851.80	907.27	96.74	0.7043
10 h	842.00	1,001.33	740.30	930.83	104.47	0.3803
SEM	98.05	84.61	84.19	55.65		
P-value	0.5733	0.1711	0.274	0.3805		
Crypt depth (μm)						
0 h	108.67	78.77^{B}	118.44	117.50^{B}	9.50	0.0572
2 h	109.13	117.43^{A}	131.40	122.10^{B}	7.78	0.3046
3 h	114.73	89.87^{AB}	145.93	124.97^{AB}	13.85	0.1065
5 h	98.53^{b}	$83.47^{AB,b}$	138.40^{a}	$133.87^{AB,a}$	5.50	0.0002
10 h	104.53	112.83^{AB}	122.90	147.73^{A}	15.40	0.2918
SEM	10.14	10.62	14.82	7.25		
P-value	0.8369	0.0897	0.691	0.0911		
Villus height:crypt depth						
0 h	7.53^{b}	10.27^{a}	6.13^{b}	6.71^{b}	0.67	0.0108
2 h	8.52	8.53	7.42	7.28	0.91	0.6502
3 h	8.59	8.66	6.19	6.79	1.01	0.2770
5 h	7.77	9.62	6.26	6.85	1.01	0.1761
10 h	8.25^{a}	9.03^{a}	6.05^{b}	6.34^{b}	0.49	0.0066
SEM	1.11	0.97	0.75	0.35		
P-value	0.945	0.7002	0.6857	0.5007		

[A,B]Means in the same column with no common superscripts differ significantly ($P < 0.05$).

[a,b]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

[1]n = 3 for each treatment group.

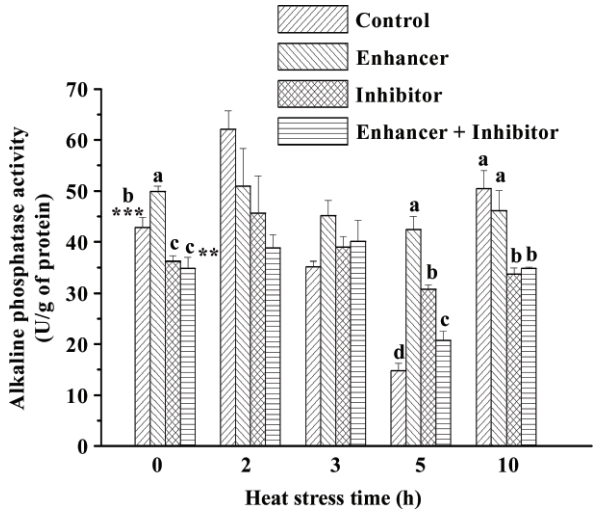

Figure 1. Relationship between the expression of heat shock protein 70 and the levels of alkaline phosphatase in the jejunal mucosa of chickens. Values were expressed as means ± SE, n = 3 for each treatment group. [a–d]Means with different letters were significantly different by Duncan multiple-range test ($P < 0.05$).**$P < 0.01$ compared with different heat-stress times in the same group; and ***$P < 0.001$ compared with different heat-stress times in the same group.

There were significant effects from different heat-stress times on the alkaline phosphatase activity in the control and the enhancer + inhibitor groups ($P < 0.01$).

Digestive Enzyme Activities

The activities of digestive enzymes in the small intestinal contents of chickens are shown in Table 4. The amylase, lipase, and trypsin activity levels in the enhancer group were significantly higher than those in the control group during the heat stress ($P < 0.001$). The amylase activities in the inhibitor group were significantly lower than that in the control during the heat stress ($P < 0.001$). The lipase activities in the inhibitor group were significantly lower than that in the control group, except after 5 h of heat stress ($P < 0.001$). The trypsin activities in the inhibitor group were significantly lower than that in the control group, except after 3 h of heat stress ($P < 0.001$). Furthermore, the amylase, lipase, and trypsin activities in the enhancer group were significantly higher than that in the inhibitor and the enhancer + inhibitor groups ($P < 0.001$). A significant effect from different heat-stress times on the digestive enzyme activities was found ($P < 0.001$).

Correlation Analysis of HSP70 and Intestinal Digestive Enzyme

Figure 2 demonstrates the correlation between HSP70 and the intestinal digestive enzyme under heat stress. At 3 h of heat stress, the amylase, lipase, and trypsin activities were positively correlated with *HSP70* expression, and the correlation coefficient reached its maximum ($P = 0.0002$, R = 0.7613; $P < 0.0001$, R = 0.7987; and $P = 0.001$, R = 0.6762, respectively). The amylase, lipase, and trypsin activities were also positively correlated with the *HSP70* mRNA expression during the heat stress. For amylase activity, the correlation coefficient was 0.6364 ($P = 0.0019$) at 5 h of heat stress. At 10 h of heat stress, the correlation coefficients for lipase

Table 4. Relationship between the overexpression of heat shock protein 70 and the intestinal digestive enzyme activity under heat stress[1]

Treatment	Control	Enhancer	Inhibitor	Enhancer + inhibitor	SEM	*P*-value
Amylase (U/dL)						
0 h	66,784.04^{AB,b}	97,183.10^{A,a}	46,244.13^{B,c}	68,661.97^{A,b}	3,997.85	<0.0001
2 h	55,868.54^{C,c}	75,821.60^{B,a}	44,718.31^{B,d}	65,727.70^{A,b}	2,270.60	<0.0001
3 h	63,615.02^{B,b}	80,633.80^{B,a}	51,291.08^{AB,c}	51,291.08^{B,c}	2,256.91	<0.0001
5 h	74,413.15^{A,b}	96,596.24^{A,a}	57,042.25^{A,c}	71,948.36^{A,b}	2,855.52	<0.0001
10 h	68,544.60^{AB,b}	76,995.31^{B,a}	48,591.55^{B,c}	69,718.31^{A,ab}	2,615.03	<0.0001
SEM	2,554.81	2,857.11	2,492.91	2,383.64		
P-value	0.0006	<0.0001	0.0152	<0.0001		
Lipase (U/L)						
0 h	582.72^{A,b}	689.70^{B,a}	418.34^{B,c}	617.51^{A,ab}	29.58	<0.0001
2 h	487.92^{B,b}	600.99^{C,a}	334.85^{C,c}	382.68^{B,c}	22.19	<0.0001
3 h	411.38^{C,b}	606.20^{C,a}	340.94^{C,c}	387.90^{B,bc}	18.76	<0.0001
5 h	416.60^{C,c}	782.76^{A,a}	576.63^{A,b}	615.77^{A,b}	25.79	<0.0001
10 h	543.58^{AB,b}	692.31^{B,a}	405.30^{BC,c}	354.85^{B,c}	18.15	<0.0001
SEM	22.31	26.84	23.15	20.43		
P-value	<0.0001	0.0003	<0.0001	<0.0001		
Trypsin (U/mL)						
0 h	1,212.44^{D,c}	1,660.22^{C,a}	1,091.89^{C,d}	1,422.56^{BC,b}	32.91	<0.0001
2 h	1,829.00^{B,b}	2,314.67^{AB,a}	1,543.11^{A,c}	1,522.44^{AB,c}	33.44	<0.0001
3 h	1,322.67^{D,c}	2,307.78^{AB,a}	1,271.00^{B,c}	1,515.56^{AB,b}	44.73	<0.0001
5 h	1,953.00^{A,b}	2,256.11^{B,a}	1,264.11^{B,c}	1,360.56^{C,c}	40.20	<0.0001
10 h	1,505.22^{C,b}	2,438.67^{A,a}	1,209.00^{B,c}	1,587.89^{A,b}	49.67	<0.0001
SEM	40.82	47.95	35.23	37.70		
P-value	<0.0001	<0.0001	<0.0001	0.0022		

[a–d]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

[A–D]Means in the same column with no common superscripts differ significantly ($P < 0.05$).

[1]n = 6 for each treatment group.

and trypsin activities were 0.7153 ($P = 0.0005$) and 0.8738 ($P < 0.0001$), respectively (Figure 3).

DISCUSSION

In the present study, the acute heat exposure (36°C) model was employed and Gln was used to establish an *HSP70* overexpression model in broiler chickens under heat stress. The results show significantly increased HSP70 and *HSP70* mRNA expression in the enhancer group during heat exposure. The current data indicate that *HSP70* overexpression does not affect the intestinal morphology under heat stress; however, there is a strong positive correlation between *HSP70* expression and the digestive enzyme activity. The overexpression of *HSP70* significantly increases the amylase, lipase, and trypsin activities under heat stress.

Heat stress is an important factor influencing domestic animal production during the summer months (Khajavi et al., 2003; Spencer et al., 2005). Heat shock protein 70 is the most conservative and the most common family in the HSP family, which is abundant in most organisms and increases synthesis after cell stress. The current research demonstrates that compared with normal conditions, the content of HSP70 mRNA and protein increase after exposure to heat stress. This result also confirms that pretreatment with Gln induces asignificant increase in *HSP70* mRNA and protein expression in the jejunal mucosa under heat stress. Our results concur with previous studies that Gln supplementation could stimulate an increase in *HSP70* protein or mRNA expression after heat stress (Ehrenfried et al., 1995; Wischmeyer et al., 2001). We established an *HSP70* overexpression chicken model by Gln.

In recent years, studies have found that there is some correlation between high-temperature stress on the chicken pathological lesion and the expression of *HSP70* (Bao et al., 2004; Sun et al., 2007). The intestine is susceptible to heat stress, hypoxia, and other environmental factors, which result in mucosal damage. Studies found that a variety of stress factors in the intestinal tract, such as endotoxins, arsenite, ethanol, and ischemia may stimulate the production of HSP70 (Beck et al., 1995; Stojadinovic et al., 1995; Tsuruma et al., 1999; Tsukimi and Okabe, 2001).

It is said that damage to the intestinal mucosal structure and digestion absorption function under heat exposure is the main factor for decreased feed intake and feed conversion (Ryder et al., 2004). Ning et al. (2003) reported that there were severe effects of heat stress on pathological damage of the duodenum, jejunum, and ileum, which mainly involved mucosal epithelial cell exfoliation and villi fracture. Studies of Liu et al. (2009) and Yu et al. (2010) found that heat stress caused marked damage to porcine intestinal epithelia, which included damage to the tips of the intestinal villi, inducing epithelial cell shedding, exposing the intestinal mucosa lamina propria, as well as shortening villus height and crypt depth. In contrast to these results, we did not find significant changes in the intestinal morphology among the birds under heat stress in our study. We think this is because heat-stress times were different and detecting morphological changes of villi and crypts requires a process. As a sensitive indicator of cell damage, lactic dehydrogenase (**LDH**) appears to significantly change before intestinal morphology change happens. There are significant increases of LDH in the jejunal mucosa during heat stress, and the overexpression of *HSP70* significantly decreases the level of LDH (data shown in the companion paper; Gu et al., 2012). This indicates that intestinal cell damage happens after exposure to heat, and *HSP70* plays an important role in the cell stress injury protection.

The results of the current study demonstrate the overexpression of *HSP70* may increase alkaline phosphatase activity. Intestinal alkaline phosphatase is the traditional marker of intestinal maturation and is located in the small intestinal epithelium villus brush border and regulated by endotoxins of intestinal normal flora (Alpers et al., 1995). It is related to intestinal digestion and absorption and plays an important role in the maintenance of normal intestinal barrier function (Bates et al., 2006). Its change may reflect the change in intestinal digestion and absorption; however, the underlying mechanism is not clear.

Changes in digestive enzyme activity may be one reason for the change in digestive function under heat stress. However, studies focused on the effects of heat stress on the poultry digestive enzyme activity are few at present. Furthermore, experimental conditions, such as heat stress mode, the duration of heat stress, and the feed and water system are different, thus the results are not consistent. The study of Ruan and Niu (2001) showed that, after 4 wk of continuous heat exposure under 34.7°C, the total proteolytic enzyme, lipase, and amylase activity in broiler intestinal contents were significantly decreased compared with that of the control group at 49 d of age. On the contrary, Routman et al. (2003) reported that pancreatic trypsin and lipase were not significantly different compared with those of the control group, but the pancreas amylase activity significantly decreased under acute heat stress. The amylase, lipase, and trypsin activity data presented here indicate that heat stress has a significant influence on the digestive enzyme activity.

To further study the relationship between changes in intestinal digestion and absorption and the expression of *HSP70* under heat stress, we measured the content of amylase, lipase, and trypsin activity in the jejunal digesta and analyzed their correlation to *HSP70* expression. The present results clearly show that there is a strong positive correlation between *HSP70* (or *HSP70* mRNA) expression and the digestive enzyme activity under heat stress. Hence, there is a possibility that the overexpression of *HSP70* may improve intestinal digestion and absorption function under acute heat stress

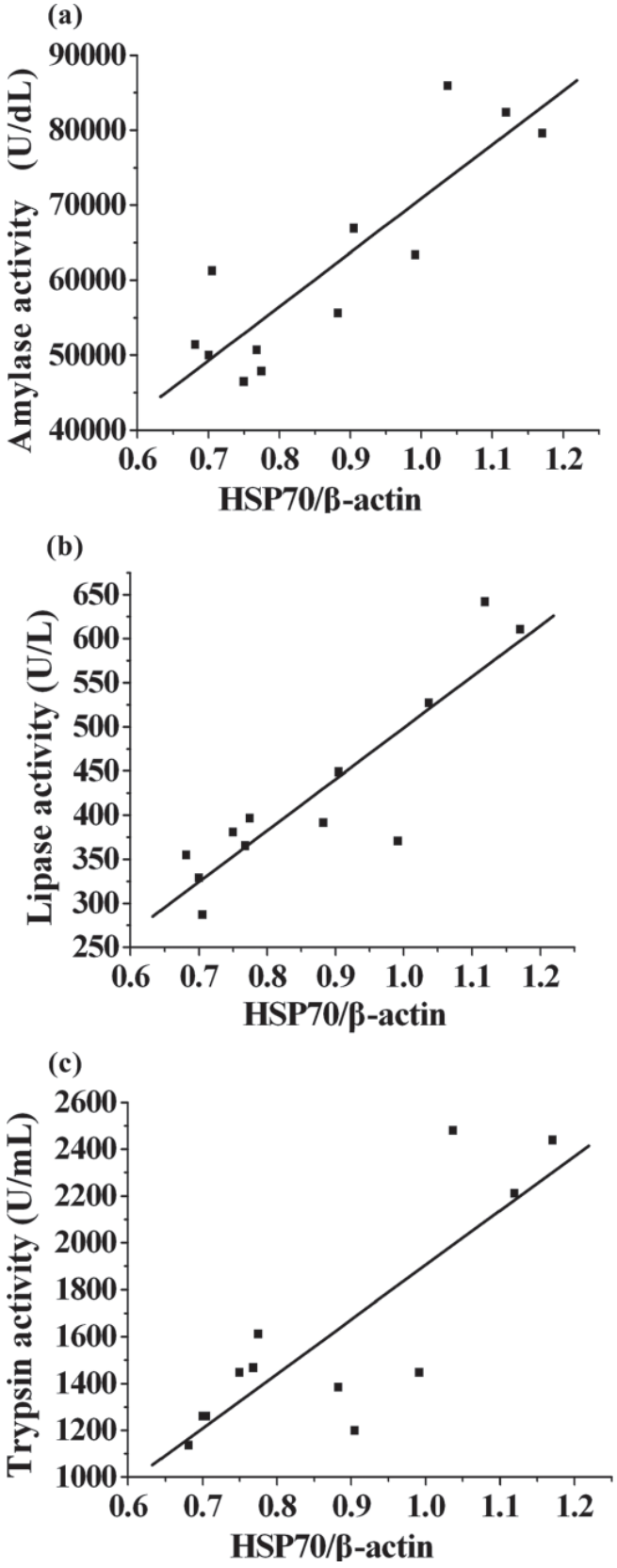

Figure 2. Correlations between heat shock protein (HSP)70 and the intestinal digestive enzyme at 3 h of heat stress. (a) HSP70 and amylase activity correlations. Regression equation was Y = 71,934X – 1,047.39; $P = 0.0002$; and R = 0.761. (b) HSP70 and lipase activity correlations. Regression equation was Y = 581.17X – 82.43; $P < 0.0001$; and R = 0.799. (c) HSP70 and trypsin activity correlations. Regression equation was Y = 2,322.52X – 417.01; $P = 0.001$; and R = 0.676. n = 12 observations for all P-values and correlation coefficients.

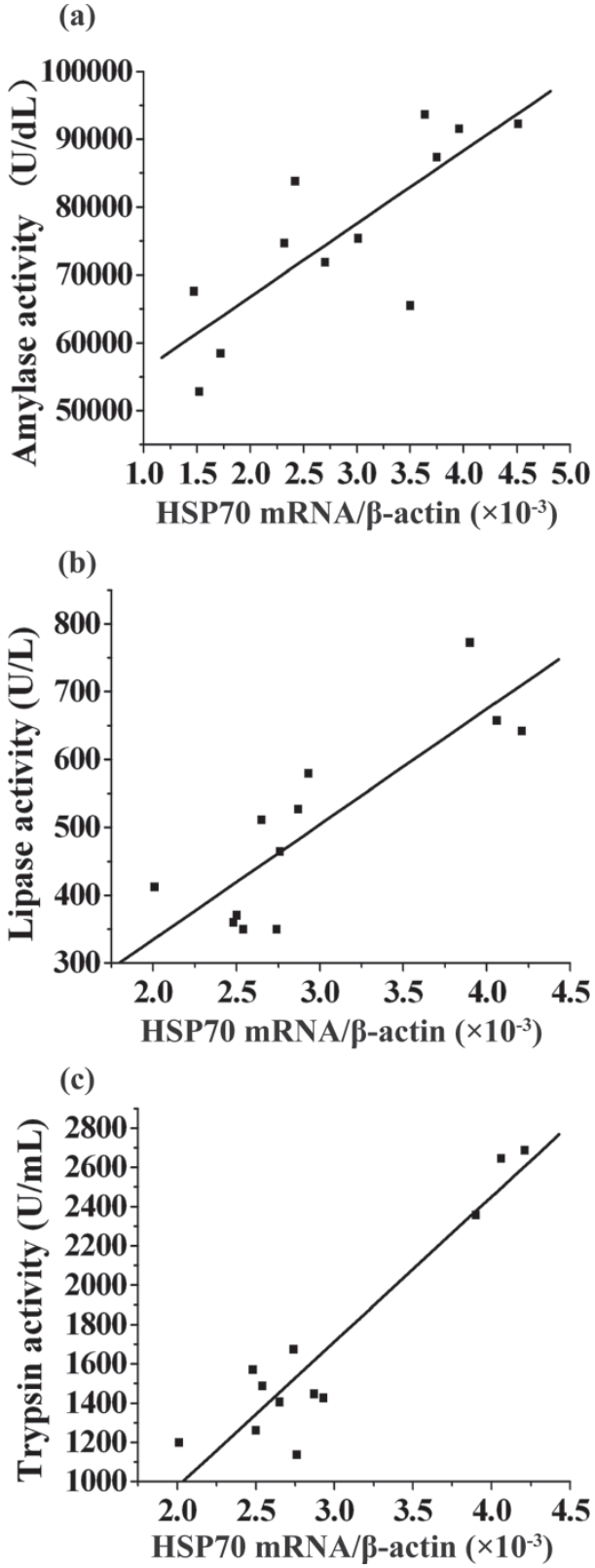

Figure 3. Correlations between heat shock protein (HSP)70 mRNA and the intestinal digestive enzyme under heat stress. (a) HSP70 mRNA and amylase activity correlations at 5 h of heat stress. Regression equation was Y = 10,740,978X + 45,345; $P = 0.0019$; and R = 0.636. (b) HSP70 mRNA and lipase activity correlations at 10 h of heat stress. Regression equation was Y = 170,158X – 5.78; $P = 0.0005$; and R = 0.715. (c) HSP70 mRNA and trypsin activity correlations at 10 h of heat stress. Regression equation was Y = 738,949X – 503.76; $P < 0.0001$; and R = 0.874. n = 12 observations for all P-values and correlation coefficients.

by increasing intestinal digestive enzyme activity. However, the physiological regulation mechanism of HSP70 on poultry digestive enzymes still needs further study.

Interestingly, we found that the strong correlation for *HSP70* only occurred after 3 h of heat stress; however, the strong correlation for HSP70 mRNA occurred from 3 to 10 h of heat stress. There appears to be no definite explanation for the phenomenon. The heat-shock response is regulated at the transcriptional level by the activities of a family of heat shock factors (**HSF**), in which HSF1 is the best-characterized and essential for the heat-shock response. In response to heat shock and other protein-damaging stresses, HSF1 undergoes nuclear localization and assembly into stress granules, becomes inducibly phosphorylated, and subsequently acquires transcriptional activity (Pirkkala et al., 2001). Based on a strong correlation between increased phosphorylation and transcriptional activity, we predicted continuous phosphorylation was one reason for the strong correlation from 3 to 10 h of heat stress. But this hypothesis needs to be evaluated rigorously in future studies. Under stress conditions, trimer formation of phosphorylated HSF-1 activates its movement into the nucleus where it binds to stimulate promoter lesion (heat shock element) and transcription is then initiated, followed by translation. The newly synthesized HSP bind to HSF to prevent further synthesis of HSP (Kiang and Tsokos, 1998). Therefore, the strong correlation only happens after 3 h.

In conclusion, our results suggest that Gln is a good *HSP70* enhancer to establish an *HSP70* overexpression model. Although the overexpression of *HSP70* does not change intestinal morphology conditions, it significantly increases broiler digestive enzyme activity under heat stress.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (grant 30871797).

REFERENCES

Al-Aqil, A., and I. Zulkifli. 2009. Changes in heat shock protein 70 expression and blood characteristics in transported broiler chickens as affected by housing and early age feed restriction. Poult. Sci. 88:1358–1364.

Alpers, D. H., Y. Zhang, and D. J. Ahnen. 1995. Synthesis and parallel secretion of rat intestinal alkaline phosphatase and a surfactant-like particle protein. Am. J. Physiol. 268:E1205–E1214.

Altan, O., A. Pabuccuoglu, A. Altan, S. Konyalioglu, and H. Bayraktar. 2003. Effect of heat stress on oxidative stress, lipid peroxidation, and some stress parameters in broilers. Br. Poult. Sci. 44:545–550.

Bao, E. D., Y. Y. Gong, J. Hartung, X. B. Fu, X. J. Wang, H. T. Zhang, and Z. L. Wang. 2004. Relation between pathologic damages and HSP_{70}, changes in acute heat-stressed broilers. Sci. Agric. Sinica 37:301–305. (In Chinese)

Basu, N., A. E. Todgham, P. A. Ackerman, M. R. Bibeau, K. Nakano, P. M. Schulte, and G. K. Iwama. 2002. Heat shock protein genes and their functional significance in fish. Gene 295:173–183.

Bates, J. M., E. Mittge, J. Kuhlman, K. N. Baden, S. E. Cheesman, and K. Guillemin. 2006. Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. Dev. Biol. 297:374–386.

Beck, S. C., C. N. Paidas, M. L. Mooney, E. A. Deitch, and A. De Maio. 1995. Presence of the stress-inducible form of hsp-70 (hsp-72) in normal rat colon. Shock 3:398–402.

Deane, E. E., and N. Y. S. Woo. 2005. Cloning and characterization of the hsp70 multigene family from silver sea bream: Modulated gene expression between warm and cold temperature acclimation. Biochem. Biophys. Res. Commun. 330:776–783.

Ehrenfried, J. A., J. Chen, J. Li, and B. M. Evers. 1995. Glutamine-mediated regulation of heat shock protein expression in intestinal cells. Surgery 118:352–356.

Gabai, V. L., A. B. Meriin, D. D. Mosser, A. W. Caron, S. Rits, V. I. Shifrin, and M. Y. Sherman. 1997. HSP_{70} prevents activation of stress kinase—A novel pathway of cellular thermotolerance. J. Biol. Chem. 272:18033–18037.

Geraert, P. A., J. C. Padilha, and S. Guillaumin. 1996. Metabolic and endocrine changes induced by chronic heat exposure in broiler chickens: Growth performance, body composition, and energy retention. Br. J. Nutr. 75:195–204.

Gu, X. H., Y. Hao, and X. L. Wang. 2012. Overexpression of heat shock protein 70 and its relationship to intestine under acute heat stress in broilers: 2. Intestinal oxidative stress. Poult. Sci. 91:790–799.

Khajavi, M., S. Rahimi, Z. M. Hassan, M. A. Kamali, and T. Mousavi. 2003. Effect of feed restriction early in life on humoral and cellular immunity of two commercial broiler strains under heat-stress conditions. Br. Poult. Sci. 44:490–497.

Kiang, J. G., and G. C. Tsokos. 1998. Heat shock protein 70 kDa: Molecular biology, biochemistry, and physiology. Pharmacol. Ther. 80:183–201.

Kojima, R., T. Tamaki, A. Kawamura, Y. Konoeda, M. Tanaka, M. Katori, N. Yokota, T. Hayashi, Y. Takahashi, and A. Kakita. 1998. Expression of heat shock proteins induced by L(1)-glutamine injection and survival of hypothermically stored heart grafts. Transplant. Proc. 30:3746–3747.

Liu, F., J. Yin, M. Du, P. Yan, J. Xu, X. Zhu, and J. Yu. 2009. Heat-stress-induced damage to porcine small intestinal epithelium associated with downregulation of epithelial growth factor signaling. J. Anim. Sci. 87:1941–1949.

Mahmoud, K. Z., F. W. Edens, E. J. Eisen, and G. B. Havenstein. 2003. Effect of ascorbic acid and acute heat exposure on heat shock protein 70 expression by young white leghorn chickens. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 136:329–335.

Ming, J., J. Xie, P. Xu, W. B. Liu, X. P. Ge, B. Liu, Y. J. He, Y. F. Cheng, Q. L. Zhou, and L. K. Pan. 2010. Molecular cloning and expression of two HSP70 genes in the Wuchang bream (*Megalobrama amblycephala Yih*). Fish Shellfish Immunol. 28:407–418.

Ning, Z. Y., S. D. Liu, D. M. Zhao, X. Tan, and S. M. Yu. 2003. The influence of heat stress on morphological and ultrastructure change of respiratory, digestive, and endocrine tissues in broilers. Acta Vet. Zootech. Sinica 34:558–561. (In Chinese)

NRC. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

Ohkawara, T., J. Nishihira, H. Takeda, T. Katsurada, K. Kato, T. Yoshiki, T. Sugiyama, and M. Asaka. 2006. Protective effect of geranylgeranylacetone on trinitrobenzene sulfonic acid-induced colitis in mice. Int. J. Mol. Med. 17:229–234.

Pirkkala, L., P. Nykänen, and L. Sistonen. 2001. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. FASEB J. 15:1118–1131.

Quinteiro-Filho, W. M., A. Ribeiro, V. Ferraz-de-Paula, M. L. Pinheiro, M. Sakai, L. R. M. Sá, A. J. P. Ferreira, and J. Palermo-Neto. 2010. Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens. Poult. Sci. 89:1905–1914.

Ren, H., M. W. Musch, K. Kojima, D. Boone, A. Ma, and E. B. Chang. 2001. Short fatty acids induce intestinal epithelial heat shock protein 25 and IEC18 cells. Gastroenterology 121:631–639.

Routman, K. S., L. Yoshida, A. C. Frizzas de Lima, M. Macari, and J. M. Pizauro Jr. 2003. Intestinal and pancreas enzyme activ-

ity of broilers exposed to thermal stress. Braz. J. Poult. Sci. 5:23–27.

Ruan, H., and D. Niu. 2001. Study on hot stress reducing activities of digestive enzymes in broiler intestine. Chinese J. Anim. Sci. 37:16–17. (In Chinese)

Ryder, A. A., J. J. R. Feddes, and M. J. Zuidhof. 2004. Field study to relate heat stress index to broiler performance. J. Appl. Poult. Res. 13:493–499.

Smith, M. O. 1993. Parts yield of broilers reared under cycling high temperatures. Poult. Sci. 72:1146–1150.

Spencer, J. D., A. M. Gained, E. P. Berg, and G. L. Allee. 2005. Diet modification to improve finishing porcine performance and pork quality attributes during periods of heat stress. J. Anim. Sci. 83:243–254.

Stojadinovic, A., J. Kiang, R. Smallridge, R. Galloway, and T. Shea-Donohue. 1995. Induction of heat-shock protein 72 protects against ischemia/reperfusion in rat small intestine. Gastroenterology 109:505–515.

Sun, P. M., Y. T. Liu, Y. G. Zhao, E. D. Bao, and Z. L. Wang. 2007. Relationship between heart damages and HSP mRNA in persistent heat-stressed broilers. Agric. Sci. China 6:227–233.

Tchernitchko, D., M. Bourgeois, M. E. Martin, and C. Beaumont. 2002. Expression of the two mRNA isoforms of the iron transporter Nramp2/DMT1 in mice and function of the iron responsive element. Biochem. J. 363:449–455.

Tsukimi, Y., and S. Okabe. 2001. Recent advances in gastrointestinal pathophysiology: Role of heat shock proteins in mucosal defense and ulcer healing. Biol. Pharm. Bull. 24:1–9.

Tsuruma, T., A. Yagihashi, N. Watanabe, T. Yajima, H. Kameshima, J. Araya, and K. Hirata. 1999. Heat-shock protein-73 protects against small intestinal warm ischemia reperfusion injury in the rat. Surgery 125:385–395.

Wischmeyer, P. E., M. D. Kahana, R. Wolfson, H. Ren, M. M. Musch, and E. B. Chang. 2001. Glutamine induces heat shock protein and protects against endotoxin shock in the rat. J. Appl. Physiol. 90:2403–2410.

Wischmeyer, P. E., M. W. Musch, M. B. Madonna, R. Thisted, and E. R. Chang. 1997. Glutamine protects intestinal epithelial cells: Role of inducible HSP70. Am. J. Physiol. 272:G879–G884.

Wu, G., S. A. Meier, and D. A. Knabe. 1996. Dietary glutamine supplementation prevents jejunal atrophy in weaned pigs. J. Nutr. 126:2578–2584.

Yu, J., P. Yin, F. H. Liu, G. L. Cheng, K. J. Guo, A. Lu, X. Y. Zhu, W. L. Luan, and J. Q. Xu. 2010. Effect of heat stress on the porcine small intestine: A morphological and gene expression study. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 156:119–128.