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Improvement in the *in vitro* Digestibility of Shrimp Meal by the Addition of Persimmon Peel

Manisa Sangkaew[1] and Katsuki Koh[2]

[1] Interdisciplinary Graduate School of Science and Technology, Shinshu University, Minamiminowa, Nagano 399−4598, Japan
[2] Faculty of Agriculture, Shinshu University, Minamiminowa, Nagano 399−4598, Japan

The present study was conducted to analyze the chemical properties of persimmon peel (PP) and the *in vitro* digestibility of shrimp meal (SM) diets containing PP. Discussions whether PP can be used as a feed additive to promote digestion of SM in chickens are also included. The chemical composition and chitinase activity of dried PP was studied. SM diets containing PP were formulated according to the 4 by 6 factorial design: 4 levels of SM (0%, 10%, 15%, and 20%)× 6 levels of PP (0%, 2%, 4%, 6%, 8%, and 10%). The *in vitro* digestibility of dry matter (IVDMD), crude protein (IVCPD), and chitin (IVCD) was also studied. PP was rich in nitrogen-free extract (NFE, about 74%) and tannin (2.8%), and the highest chitinase activity of PP was observed at pH 4.5. Approximately 50% of chitinase activity was also observed at acidic (3.0) and alkaline (8.0) pH. Its activity was slightly affected by pepsin treatment. IVDMD increased upon addition of up to 8% PP, but decreased with an increase in the level of SM. When PP level was increased up to 6%, IVCPD in the group containing 0% SM, changed slightly; however, an increasing trend was observed in the other groups. When PP level was more than 6%, IVCPD decreased in all the groups. IVCD increased dose-dependently with increasing level of PP and decreased with increasing level of SM. In conclusion, PP was rich in NFE, had high chitinase activity, and improved all digestibility parameters, such as IVDMD, IVCPD, and IVCD, in SM diets where the PP level was under 6%. Thus, up to 6% of PP can be safely included in SM diets as a digestion promoter.

Key words: chitinase activity, *in vitro* digestibility, persimmon peel, shrimp meal

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Introduction

Many studies have been conducted to use shrimp meal (SM), a by-product of shrimp processing, as a protein source for poultry diets, but its practical use seems to be difficult, because of the low digestibility of chitin present in SM (Khempaka *et al*., 2006a, 2006b; Rahman and Koh, 2016a). Rahman and Koh (2016b, 2018) reported that treatment with formic acid led to a reduction in the level of chitin present in SM and improved the digestibility of SM. They also demonstrated that treated SM could replace soybean meal (SBM) present in the chicken diet by up to 15%. However, an eco-friendly method to enable organic poultry production needs to be considered. Chitinase is an enzyme that degrades chitin. Chitinase is present in plants, bacteria, and other organisms (Broekaert *et al*., 1988; Kuddus and Ahmad, 2013). It is a defense mechanism in plants against attack by chitinous organisms, such as fungi and insects (Taira *et al*., 2002; Lawrence and Novak, 2006; Wang *et al*., 2012). Among plant species, persimmon fruits have been reported to have high chitinase activity (Takii *et al*., 2010), and the peel of the astringent-type persimmon (PP) is a by-product obtained after processing of dried persimmon and is available in large amounts in the production areas, such as Nagano prefecture, which is where our faculty is located. Therefore, PP can be used for degradation of chitin present in SM. However, a concern to use PP as a feed ingredient for chicken diets arises because of the high level of tannin present, which is a factor that could lead to decreased digestibility in animals (Iji *et al*., 2004; Mariscal-Landín *et al*., 2004). However, not much information on the use of PP as a diet for chickens has been reported.

The purpose of the present study was to investigate the

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Correspondence: Dr. Katsuki Koh, Faculty of Agriculture, Shinshu University, Minamiminowa, Nagano 399−4598, Japan.
(E-mail: kkkohss@shinshu-u.ac.jp)

chemical properties of PP and the *in vitro* digestibility of SM diets containing PP. Moreover, whether PP can be used as a feed ingredient to improve the digestibility of SM has been discussed.

Materials and Methods

Preparation of PP and SM

Sun-dried PP was purchased from three different places in Nagano prefecture in Japan, such as Ijima-machi, Matsukawa-machi, and Iida city. The sun-dried PP were ground so as to pass through a 1.0 mm aperture for further processing. SM was prepared as follows: whiteleg shrimps (*Litopenaeus vannamei*) were purchased commercially from India, Japan, and Thailand in their frozen forms, thawed under running water, and then peeled. The peel wastes, such as the head and hull, were dried in an oven at 55℃ for 10 h, ground to pass through a 1.0 mm aperture, and then used as SM.

Chemical Analysis and Enzyme Assay

Proximate compositions, acid detergent fiber (ADF), and neutral detergent fiber (NDF) of SM and PP were analyzed according to the standard method (AOAC, 1990). Chitin was measured using the method described by Ghanem *et al.* (2003). Tannin was measured by the Folin-Denis method, a method which was used for persimmon tannin analysis in previous studies (Yamada *et al*., 2002; Park *et al*., 2004): briefly, the sample which was extracted with 80% methanol (5 mL) was mixed with Folin-Denis reagent (5 mL), incubated at room temperature for 3 min, post which the sodium carbonate solution (5 mL) was added. Then, after 1 h, the absorbance was measured at 760 nm, and the amount of tannin was calculated as tannic acid equivalents. Chemical compositions of SM and PP are shown in Table 1. Crude chitinase fraction of PP was obtained as follows (Koh and Iwamae, 2013): briefly, PP homogenized with the buffer having the desired pH was centrifuged, and the supernatant was salted out with 80% ammonium sulfate. The precipitate was dialyzed against the extraction buffer and centrifuged. The supernatant was measured for protein content by CBB dye-binding method (Bradford, 1976) and then used as the crude chitinase faction. Chitinase activity was measured as follows (Hirano, 1991): briefly, the reaction mixture composed of crude enzyme fraction and 1% (w/v) colloidal chitin was incubated at 37℃ for 60 min. The reaction was stopped by adding 10% tungstate and 2/3 N sulfuric acid, post which the reaction mixture was centrifuged, and the reducing sugar in the supernatant was measured according to the modified Schales method (Imoto and Yagishita, 1971). One unit (U) of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of *N*-acetylglucosamine per min at 37℃.

Effect of pH and Pepsin Resistance of PP Chitinase

The effect of pH on PP chitinase was studied using 50 mM (final concentration) of citrate or sodium phosphate buffers ranging from pH 3.0 to 8.0 at 37℃ for 60 min. Citrate buffer was used for pH 3.0−6.0 and sodium phosphate buffer for pH 6.5−8.0 (Zhang *et al*., 2013). The pepsin resistance of PP chitinase was studied using 50 mM glycine-HCl buffer at pH 2.0 containing 0 mg pepsin/mL, 5 mg pepsin/mL, and 10 mg pepsin/mL (10,000 U/mg protein, Nacalai Tesque Inc., Kyoto, Japan) at 37℃ for 60 min (Esmaeilipour *et al*., 2012). Chitinase activity was measured, as mentioned earlier.

Experimental Diets and in vitro Digestibility Measurement

Twenty-four diets were formulated according to the 4 by 6 factorial design: 4 levels of SM (0%, 10%, 15%, and 20%) × 6 levels of PP (0%, 2%, 4%, 6%, 8%, and 10%) (Table 2). PP and SM were added at the expenses of maize and SBM, respectively. Three diets were made in each combination because PP obtained from three different origins and SM obtained from a single origin (from Thailand) was used. SM obtained from Thailand was used in the *in vitro* digestibility study because there was a slight difference in the nutritional value among the SM obtained from three different sources, and SM from Thailand was supplied abundantly. All diets were formulated to meet or exceed the nutrient requirement of laying hens recommended by NRC (1994): chitin nitrogen (N) was not included while calculating the crude protein (CP) content, because of lack of evidence of utilization of chitin N in birds.

In vitro digestibility was measured by according to the method described by Saunders *et al*. (1973) with slight modi-

Table 1. **Chemical composition of shrimp meal and persimmon peel**[1]

Chemical composition, % of DM	Shrimp meal	Persimmon peel
Crude protein	51.3±2.1	4.3±0.3
Ether extract	5.6±0.9	1.6±0.1
Crude fiber	15.6±0.7	17.2±1.2
Crude ash	27.1±1.9	3.3±0.4
Nitrogen free extract (NFE)	0.4±0.1	73.6±1.6
Neutral detergent fiber (NDF)	46.0±1.0	32.7±2.1
Acid detergent fiber (ADF)	19.1±0.3	30.7±1.4
Chitin	14.7±1.3	—
Tannin	—	2.8±0.5

[1] Values of each parameter represent the mean±standard error values with three observations (dry matter basis).

Table 2. **Composition and nutrient level of the basal diet (as-fed basis)**

Diets		Ingredients[1,2], g/kg							
		Maize	PP	SBM	SM	Corn oil	$Ca_3(PO_4)_2$	$CaCO_3$	Chitin[3]
SM0%	PP0%	503	0	323	0	35	35	85	0
	PP2%	483	20						
	PP4%	463	40						
	PP6%	443	60						
	PP8%	323	80						
	PP10%	403	100						
SM10%	PP0%	492	0	235	100	47	35	72	14
	PP2%	472	20						
	PP4%	452	40						
	PP6%	432	60						
	PP8%	412	80						
	PP10%	392	100						
SM15%	PP0%	489	0	190	150	53	30	69	21
	PP2%	469	20						
	PP4%	449	40						
	PP6%	429	60						
	PP8%	409	80						
	PP10%	389	100						
SM20%	PP0%	484	0	145	200	59	29	64	28
	PP2%	464	20						
	PP4%	444	40						
	PP6%	424	60						
	PP8%	404	80						
	PP10%	384	100						

[1] Vitamin-mineral premix provided with the following concentrations per kg of diet: vitamin A, 10,500 IU; vitamin D3, 2,100 IU; vitamin E, 15 mg; thiamine, 10 mg; riboflavin, 7 mg; pantothenic acid, 15 mg; pyridoxine, 3 mg; niacin, 32 mg; choline chloride, 500 mg; folic acid, 0.6 mg; biotin, 0.1 mg; manganese, 75 mg; iron, 50 mg; zinc, 60 mg; copper, 5 mg; iodine, 2 mg. This mixture was added to all diets at the level of 19 g/kg.
[2] All diets contained 176 g/kg of CP, 11.7 MJ/kg of ME and 41 g/kg of Ca (analyzed value, as fed basis). SM=shrimp meal, PP=persimmon peel, SBM=soybean meal.
[3] Measured value.

fication: briefly, approximately 250 mg of each diet was suspended in 15 mL of 0.1 N HCl containing 1.5 mg pepsin (concentration was the same as that used in the pepsin resistance experiment) and gently shaken at 41℃ for 3 h. After neutralization with 0.5 N NaOH, this was mixed with 7.5 mL of phosphate buffer (pH 8.0) containing pancreatin (3,220 U/g of amylase, 38,500 U/g of protease, and 1,600 U/g of lipase) (Nacalai Tesque Inc.) and shaken at 41℃ for 24 h. The mixture was then centrifuged, washed with distilled water, filtered, and dried. Dry matter (DM), CP and chitin contents in the dried digesta were measured as mentioned earlier for measurement of *in vitro* digestibility of DM (IVDMD), CP (IVCPD), and chitin (IVCD).

Statistical Analysis

Statistical significance among the various treatments was determined using Tukey's multiple comparison tests at a significance level of 5%. The *in vitro* digestibility data were analyzed by the two-way ANOVA using the general linear model (GLM) procedure (SAS Institute, 2015).

Results

Chemical Properties of SM and PP

The major proximate composition of SM was CP which was approximately 50% of the total composition. The second and third major components were crude ash and crude fiber, respectively. The other components were present at lower than 6% of the total composition. In SM, the value of NDF was 46%, which was more than two times greater than the value of ADF (Table 1). Approximately 15% of chitin was present in SM. On the contrary, the major proximate composition of PP was NFE which was approximately 74% of the total composition, and the second major component was crude fiber. The other components were present at lower than 5%. In PP, both NDF and ADF were present at approximately 30%, and tannin was present at approximately 3%. Maximum PP chitinase activity was obtained (about 1.30 U/mg of protein) at pH 4.5, and 50% activity was obtained at pH values ranging from 3.0 to 8.0 (Fig. 1, panel A). PP chitinase activity at pH 2.0 containing no pepsin was about 0.58 U/mg of protein, which changed slightly in the

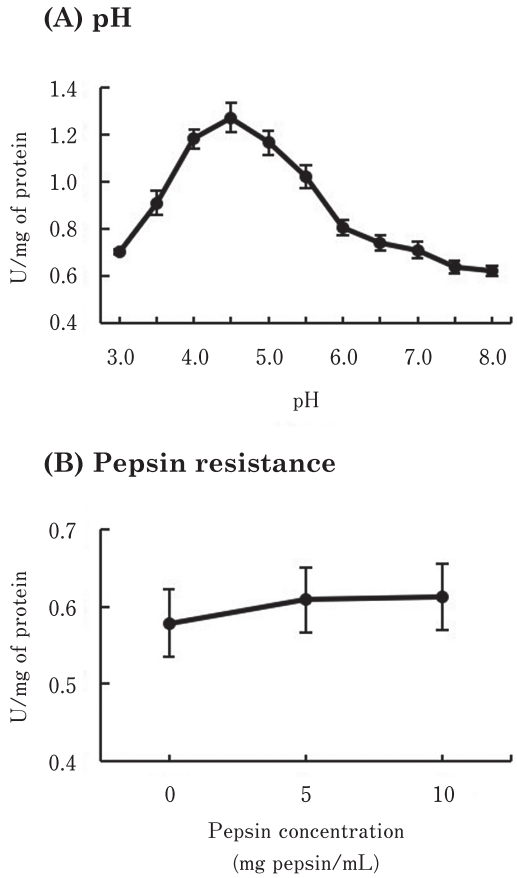


Fig. 1. **pH behavior and pepsin resistance of persimmon peel chitinase.** Data represent mean±SEM (n=3). One unit (U) of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of *N*-acetylglucosamine per min at 37℃.

presence of 5 mg/mL and 10 mg/mL pepsin in the buffer (Fig. 1, panel B).

Effects of PP on Digestibility of SM Diets (Fig. 2)

IVDMD in group containing 0% SM was approximately 78% when no PP was present. IVDMD increased until the amount of PP added reached to 8% and then decreased. A similar pattern was found in other groups containing SM, but the values decreased overall with increasing amounts of SM. Results of two-way ANOVA showed that IVDMD was affected by both PP and SM significantly, but their interaction was not significant.

IVCPD in the group containing 0% SM was approximately 72% when no PP was present, and it changed slightly until the amount of PP added reached to 6% and then decreased. On the contrary, IVCPD in the other SM groups increased until the amount of PP added reached to 6% and then decreased. With an exception of the group containing 0% SM, IVCPD decreased with an increase in the level of SM. Results of two-way ANOVA showed that IVCPD was affected by both PP and SM significantly, and their interaction was also significant.

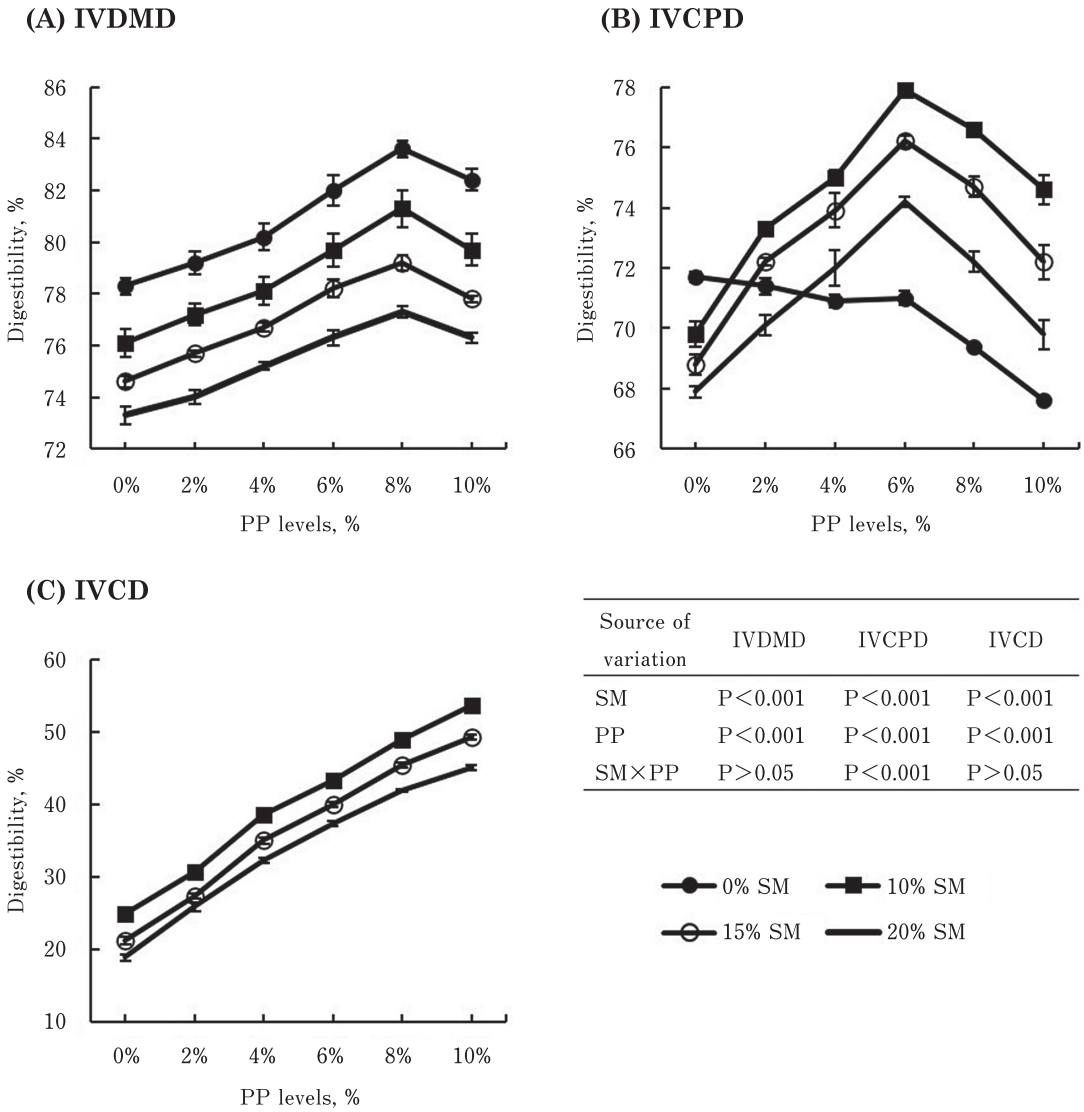
IVCD in the groups containing no PP ranged from 18.8% (in the group containing 20% SM) to 24.9% (in the group containing 10% SM). As were the cases with IVDMD and IVCPD in the SM containing groups, IVCD increased with an increase in the level of PP and decreased with an increase in the amount of SM. However, no decrease was observed even when the amount of PP added was above 8%. Results of two-way ANOVA showed that IVCD was affected by both PP and SM significantly, but their interaction was not significant.

Discussion

This is the first report on the promoting effect of PP on the digestion of dietary SM. Moreover, SM used in the present study was rich in CP (about 50%), and the standard variation of each chemical composition in SM was very small, suggesting that nutritional aspects of SM were similar among regions of different origins, such as India, Japan, and Thailand. PP was rich in NFE (about 74%), and thus, it could be used as an energy source, although the presence of approximately 17% of crude fiber may be a concern. In PP, 2.8% of tannin was present, which may be an applicable level, because sorghum, widely used as a poultry feed ingredient, contains various levels of tannin, sometimes as high as 3.9% of tannin (Elkin *et al*., 1996). Maximum activity for PP chitinase was obtained at pH 4.5, and interestingly, relatively high activity (approximately 50%) was obtained at acidic (3.0) and alkaline (8.0) pH. Moreover, PP chitinase did not lose its activity even in the presence of buffer containing 10 mg/mL pepsin. Thus, PP chitinase is expected to function not only in the crop but also in the proventriculus and gizzard of chickens.

IVDMD in the group containing 0% SM increased with an increase in the level of PP i.e. from 0% PP to 8% PP. However, it decreased when the amount of PP was increased to 10%. This pattern may be explained as follows: increase in the IVDMD may be due to higher digestibility in PP than in maize because PP in the group containing 0% SM was replaced with maize. Moreover, a decrease in the IVDMD may be due to an increase in the level of tannin. Tannins are well known to form complexes with proteins, starch, and digestive enzymes, and thus, they reduce the nutritional values of food (Chung *et al*., 1998). Patterns of IVDMD in other groups with different percentages of SM were similar to that in group containing 0% SM. No significant interaction between PP and SM levels was observed. Consequently, the pattern of IVDMD in all groups may be regulated by a common mechanism. On the contrary, an overall decrease in the IVDMD with increasing level of SM was observed, which may be due to poor digestibility of SM. The IVDMD of SM obtained from whiteleg shrimp was reported to be approximately 61.5% (Rahman and Koh, 2014).

When the amount of PP added was increased from 0% to 6%, the IVCPD in the group containing 0% SM changed slightly. However, the IVCPD in the groups containing 10%, 15%, and 20% of SM increased. This could be due to complete digestion of the protein obtained from the non-SM



Source of variation	IVDMD	IVCPD	IVCD
SM	P<0.001	P<0.001	P<0.001
PP	P<0.001	P<0.001	P<0.001
SM×PP	P>0.05	P<0.001	P>0.05

Fig. 2. ***In vitro* digestibility of dry matter (IVDMD), crude protein (IVCPD), and chitin (IVCD) in shrimp meal diet containing persimmon peel.** PP=persimmon peel, SM=shrimp meal. Data represent mean±SEM (n=3).

origin with proteolytic enzymes, such as pepsin and protease in pancreatin, in the buffer, and limited or incomplete digestion of protein obtained from the SM origin by similar enzymes in the absence of PP chitinase. Therefore, PP may be a promising component to improve the digestibility of CP in SM. However, the IVCPD decreased not only in the group containing 0% SM, but also in other groups containing 10%, 15%, and 20% of SM, when the amount of PP added was increased to more than 6%. This could suggest that the digestibility of protein obtained from both the SM and non-SM origins were impaired by the tannin level in diets containing PP at 6% or more. The tannin level in the diets containing 6% PP was 0.17%. The presence of tannin leading to a decrease in *in vivo* digestibility of CP and amino acids are different among reports, such as 2.5% (Iji *et al*., 2004) and 0.41% (Woyengo and Nyachoti, 2012). However, the percentage of tannin reported in literature is higher than the percentage obtained in the present study. This difference may be due to an increase in the pancreatic enzyme secretion after tannins bind to the digestive enzymes (Griffiths and Moseley, 1980).

About 20% of IVCD was recorded even in the group containing 0% PP, which indicates that there is another factor involved in the digestion of chitin besides PP chitinase. Feed ingredients might not be the factor, because, on comparing with PP, very low chitinase activity was found in maize (0.19 U/mg of protein) (Huynh *et al*., 1992) and soybean (0.46×10^{-3} U/mg of protein) (Wadsworth and Zikakis, 1984). How-

ever, its activity in SBM may be lower than that because of heating. High chitinase activity in SM may not be expected, because an increased level of SM leads to a decrease in IVCD. Moreover, pepsin in the buffer can be the factor, because there is a report showing that this enzyme not only hydrolyzes protein but also chitin (Ilankovan *et al*., 2006). IVCD in all SM groups increased linearly with an increase in the level of PP, indicating that PP chitinase digested chitin in SM dose-dependently. However, unlike the results of IVDMD and IVCPD, IVCD did not decrease even when the amount of PP added reached 10%, suggesting that chitinase in PP was not affected by tannin. This might be true, because tannin-sensitive chitinase may not be able to function in persimmon fruits, as the role of plant chitinase is recognized as a defense mechanism against chitin-containing organisms (Zhang *et al*., 2013).

In conclusion, the present study revealed that PP was rich in NFE, had a high chitinase activity, and improved all digestibility parameters, such as IVDMD, IVCPD, and IVCD, of SM diets when the amount of PP added was under 6%. This suggests that up to 6% of PP can be safely included in SM diets as a digestion promoter.

Conflicts of Interest

The authors declare no conflict of interest.

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