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Original Research Article

Anti-Oxidative, Metal Chelating and Radical Scavenging Effects of Protein Hydrolysates from Blue-spotted Stingray

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Abstract

Purpose: *To evaluate protein hydrolysates and membrane ultrafiltration fractions of blue-spotted stingray for metal chelating and radical scavenging activities, as well as protection against oxidative protein damage.*

Methods: *Stingray protein isolates were hydrolysed with alcalase, papain and trypsin for 3 h. Alcalase hydrolysate was fractionated by membrane ultrafiltration to yield < 3, 3 - 10 and > 10 kDa fractions. Peptide contents, iron and copper chelating activity, 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and hydroxyl radical scavenging activities, and protection against oxidative protein damage were evaluated.*

Results: *Three-hour alcalase hydrolysate (3AH) had the highest peptide content and the lowest half maximal effective concentration (EC_{50}) for ABTS radical scavenging (793.9 μg/mL), hydroxyl radical scavenging (6.93 mg/mL), iron chelating (116.4 μg/mL) and copper chelating activity (2136.9 μg/mL) among the hydrolysates. Among the fractions of 3AH, < 3 kDa fraction had the best iron chelating activity, 3 - 10 kDa fraction exhibited the highest ABTS radical scavenging activity, while > 10 kDa fraction showed the best copper chelating activity. The < 3 kDa and 3 - 10 kDa fractions had similar levels of hydroxyl radical scavenging activity to reduced glutathione. The protective effects of 3AH and < 3 kDa fraction against oxidative protein damage were comparable to that of reduced glutathione.*

Conclusion: *Alcalase is the best protease for producing hydrolysates with metal chelating and antioxidant activities from stingray proteins. Alcalase hydrolysate, specifically its < 3 kDa fraction, has potential for future applications in antioxidant therapy and health food formulation.*

Keywords: *Dasyatis kuhlii, Membrane ultrafiltration, Protein hydrolysate, Glutathione, Peptide content, Metal chelating, Radical scavenging*

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INTRODUCTION

In recent years, the antioxidant potential of protein hydrolysates derived from edible fishes and other animal sources has generated great interests among researchers [1-3]. Proteases of plant, animal and microbial origins, such as papain, trypsin, chymotrypsin, neutrase, and alcalase, have been used in the production of antioxidative fish protein hydrolysates (FPH) [2,3]. The antioxidant capacity of FPH has been demonstrated through free radical scavenging, metal chelating, lipid peroxidation inhibition and other antioxidant assays [3]. The potential applications of FPH, including their use as

antioxidants to extend the shelf-life of meat products as well as their health-promoting and disease-preventive effects, have been discussed in recent reviews [2,3]. To date, most studies have focused on edible, bony marine fishes, such as salmon and tuna. Very little attention was given to the antioxidant potential of protein hydrolysates prepared from edible, cartilaginous marine fishes, such as stingrays.

The objective of this study was to assess the antioxidant capacity of protein hydrolysates which were produced from the blue-spotted stingray by enzymatic hydrolysis using alcalase, papain and trypsin. Blue-spotted stingray was chosen because the antioxidant capacity of the protein hydrolysates of this edible, commercial fish species has never been previously investigated. The three hydrolysates and peptidic fractions derived from alcalase hydrolysate via membrane ultrafiltration were tested for their metal chelating and free radical scavenging activities, as well as their ability to protect against oxidative protein damage.

EXPERIMENTAL

Protein isolation from fish sample

Blue-spotted stingray (*Dasyatis kuhlii*, Family Dasyatidae) was purchased from a local market in Kampar, Malaysia, in May 2014. The species of the fish was identified by Mr Yusri Yusuf, a fish taxonomist of the Institute of Oceanography and Environment, Universiti Malaysia Terengganu. The fish sample was eviscerated and cut into small chunks. Protein isolates were prepared from the fish sample by using the alkaline pH-shift method as previously described [4], with slight modifications. The protein pellet collected was used for hydrolysate preparation as described below. Protein content of the protein isolate was determined using Bradford's method [5].

Preparation of fish protein hydrolysates

Protein hydrolysates were prepared from the stingray protein isolate by enzymatic hydrolysis using three selected proteases: alcalase, papain and trypsin. The protein isolates were separately dissolved in 100 mM sodium phosphate buffer, adjusted to pH 8.5 for alcalase hydrolysis; to pH 7.0 for papain hydrolysis, and to pH 8.0 for trypsin hydrolysis. The proteases were added at the ratio of 1 g of enzyme to 10 g of protein. Hydrolysis was carried out at the respective optimum temperatures of each protease, namely 60 °C for alcalase and 37 °C for papain and trypsin. Hydrolysates were collected at 0 h and after 3 h of hydrolysis. The 0-h hydrolysate was taken as treatment control; it was collected immediately after the addition of a protease to the protein isolate-containing phosphate buffer. The 0- and 3-h hydrolysate samples collected were immediately heated in boiling water for 15 min to inactivate the proteases. The hydrolysates were then freeze-dried and stored at -20 ºC until used.

Preparation of peptide fractions by membrane ultrafiltration

The 3-h alcalase hydrolysate was fractionated by size through a two-step centrifugal ultrafiltration process using membranes having molecular weight cut-off (MWCO) of 3 and 10 kDa. The < 3 kDa, 3 - 10 kDa and > 10 kDa fractions obtained were freeze-dried and stored at -20 °C until used.

Determination of peptide contents

Peptide contents of hydrolysates and membrane ultrafiltration fractions were determined by using o-phthaldialdehyde (OPA) reagent [1]. Casein peptone was used to generate a standard curve for the assay.

Determination of metal chelating and radical scavenging activity

Iron chelating activity of hydrolysates and ultrafiltration fractions was evaluated as outlined in [6]. Copper chelating activity was determined as described in [7], with minor modifications. EDTA disodium was used as positive control in both assays. 2, 2'-azino-bis(3-ethylbenzothiazo-line-6-sulphonic acid) (ABTS) radical scavenging activity was determined as described in [8]. Hydroxyl radical scavenging activity was assessed for as outlined in [9], with minor modifications. Reduced glutathione, a naturally occurring tripeptide antioxidant [10] was used as positive control in ABTS radical and hydroxyl radical scavenging assays.

Protective effect against oxidative protein damage

The ability of the stingray hydrolysates and ultrafiltration fractions to protect bovine serum albumin (BSA) from *in vitro* oxidative damage was assessed as outlined in [11]. Visualisation of BSA integrity following exposure to Fenton reagent was carried out by using a sodium dodecyl sulphate-polyacrylamide gel [12]. To determine the relative amount of BSA represented by each band, intensities of the

bands were quantified using the ImageJ software (version 1.46, National Institutes of Health, Bethesda, MD, USA). The density of each band was standardised relative to the control band.

Data analysis

Experiments were carried out in triplicates. Data presented are mean ± standard error of the mean (SEM). Statistical analyses were performed using Statistical Analysis System software (version 9.2). Data were analysed by one-way ANOVA test and means of significant differences were separated using Fisher's least significant difference (LSD) test or Student's t-test at α = 0.05. EC_{50}, defined as the sample concentration required to achieve 50 % activity, was determined by using linear regression analysis.

RESULTS

Alcalase, papain and trypsin were successfully used to produce hydrolysates having enhanced peptide contents from blue-spotted stingray protein isolates (Table 1). After 3 h of protease treatments, the resulting 3 h alcalase, papain, and trypsin hydrolysates (3AH, 3PH, and 3TH) had 2.6-, 2.8-, and 2.2-fold increases respectively in peptide contents compared with their respective 0-hour hydrolysates (0AH, 0PH, and 0TH). Notably, among the 3 h hydrolysates, 3AH had the highest peptide content, which was about 2-fold higher compared to 3PH and 3TH.

For iron chelating activity, the EC_{50} of 3AH decreased by 35 % compared with 0AH (Table 1). In contrast, 3TH had a higher EC_{50} value relative to 0TH, whereas 0PH and 3PH had similar EC_{50} values. For iron chelating activity, the EC_{50} of 3AH was 3.4-fold higher than that of EDTA disodium. For copper chelating activity, 3 h hydrolysates generally had lower EC_{50} values compared with 0 h hydrolysates. 3AH had the lowest EC_{50} value among all hydrolysates, which was 2.6-fold and 2.9-fold lower compared with 3PH and 3TH. EC_{50} of 3AH was 18-fold higher than that of EDTA disodium. Similar to copper chelating activity, the ABTS radical scavenging activity of 3AH, 3PH and 3TH was enhanced, as indicated by their lower EC_{50} values compared with 0AH, 0PH and 0TH. For ABTS radical scavenging activities, the EC_{50} of 3AH was the lowest among all hydrolysates, although it was 16-fold higher compared with reduced glutathione. Based on EC_{50} values, 3AH exhibited the strongest iron chelating, copper chelating and ABTS radical scavenging activities among all three protein hydrolysates analysed.

Three membrane ultrafiltration fractions were obtained: < 3 kDa, 3 - 10 kDa, and > 10 kDa fractions. The 3 - 10 kDa fraction had the highest peptide content, whereas the > 10 kDa fraction had the lowest (Table 2). Among the three ultrafiltration fractions, the < 3 kDa fraction had the highest iron chelating activity (Table 2). The EC_{50} of < 3 kDa fraction was 53 % and 45 % lower than those of 3 - 10 kDa and > 10 kDA fractions, respectively.

When compared to 3AH (Table 1) and EDTA disodium, the EC_{50} of the < 3 kDa fraction was 2.6-fold and 8.8-fold higher, respectively. For copper chelating activity, > 10 kDa fraction had the lowest EC_{50} value among the three fractions; the EC_{50} value was also 31 % lower compared with 3AH. When compared with EDTA disodium, the EC_{50} of > 10 kDa fraction was 12-fold higher.

Table 1: Peptide contents and antioxidant activities of protein hydrolysates

Hydrolysate	Peptide content (mg/g of freeze-dried hydrolysate)	EC_{50} (µg/mL)		
		Iron chelating activity	***Copper chelating activity***	***ABTS radical scavenging activity***
0AH	169.1 ± 3.0[a]	177.8 ± 0.4*	6131.0 ± 160.2*	1437.3 ± 47.7*
3AH	433.9 ± 3.9[b]	116.4 ± 0.8*	2136.9 ± 13.9*	793.9 ± 16.6*
0PH	77.5 ± 1.9[c]	211.9 ± 3.8*	44944.8 ± 14437.5*	2115.9 ± 15.9*
3PH	213.5 ± 4.0[d]	212.2 ± 2.1*	5515.4 ± 235.6*	1132.6 ± 16.9*
0TH	94.1 ± 1.0[e]	137.8 ± 6.8*	ND	2381.3 ± 19.6*
3TH	203.7 ± 0.6[f]	162.6 ± 7.6*	6281.4 ± 616.1*	1335.9 ± 11.3*
Positive control	-	33.9 ± 0.9 (EDTA disodium)	121.3 ± 0.2 (EDTA disodium)	51.2 ± 0.5 (Reduced glutathione)

Data are presented as mean ± SEM (n=3). EC_{50} denotes the sample concentration required to achieve 50 % activity. 0AH = 0 h alcalase hydrolysate; 3AH = 3 h Alcalase hydrolysate; 0PH = 0 h Papain hydrolysate; 3PH = 3 h Papain hydrolysate; 0TH = 0 h trypsin hydrolysate; 3TH = 3 h Trypsin hydrolysate. ND, activity undetectable. For peptide content data, values followed by different superscripts (a-f) are significantly different (p < 0.05) as determined by Fisher's LSD test. For EC_{50} data, asterisks (*) indicate mean values that are significantly different (p < 0.05) from that of the positive control, as determined by Student's t-test

For ABTS radical scavenging activity, the 3 - 10 kDa fraction had the lowest EC_{50} value, followed by < 3 kDa and then > 10 kDa fraction. When compared with reduced glutathione, the EC_{50} of > 10 kDa fraction was 9.7-fold higher. The EC_{50} for the 3 - 10 kDa fraction was 37 % lower than that of 3AH. Based on EC_{50} values, the < 3 kDa fraction had the strongest iron chelating activity; the > 10 kDa fraction had the strongest copper chelating activity; and the 3 -10 kDa fraction had the strongest ABTS radical scavenging activity.

To find out if the 3 h hydrolysates and membrane ultrafiltration fractions also possessed scavenging activity against biologically-relevant free radicals, we have tested them for hydroxyl radical scavenging activity. All 3 h hydrolysates exhibited concentration-dependent increase in hydroxyl radical scavenging activity within the concentration range tested (Fig 1A). 3AH (EC_{50} = 6.93 mg/mL) showed higher hydroxyl radical scavenging activity than 3PH (EC_{50} = 9.59 mg/mL) and 3TH (EC_{50} = 9.34 mg/mL). For the ultrafiltration fractions prepared from 3AH (Fig 1B), we found the < 3 kDa and 3 - 10 kDa fractions to have similar hydroxyl radical scavenging activities, both being higher compared with the > 10 kDa fraction. The EC_{50} values of the three fractions were 4.01, 3.94 and 11.38 mg/mL, respectively. The EC_{50} values of < 3 kDa and 3 - 10 kDa fractions were not statistically different (*p* > 0.05) from that of reduced glutathione (EC_{50} = 4.11 mg/mL), as determine by student's t-test.

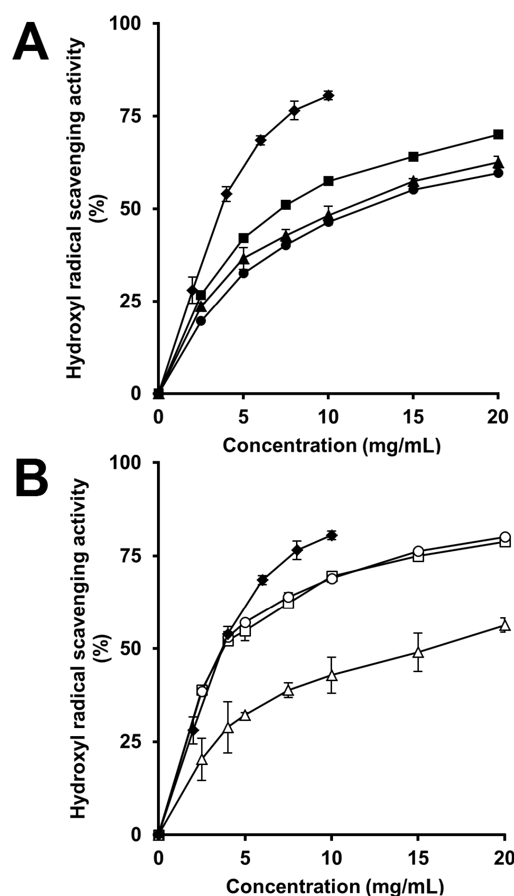

Fig 1: Hydroxyl radical scavenging activity of (A) 3 h hydrolysates (3AH (■), 3PH (●), and 3TH (▲)) and (B) ultrafiltration fractions (<3 kDa (□), 3-10 kDa (o), >10 kDa (Δ)). Reduced glutathione (♦). Data points are mean ± SEM (n=3)

Table 2: Peptide contents and antioxidant activity of membrane ultrafiltration fractions obtained from 3AH

Fraction	Peptide content (mg/g of freeze-dried fraction)	EC_{50} (µg/mL) Iron chelating activity	Copper chelating activity	ABTS radical scavenging activity
<3 kDa	470.2 ± 3.9[a]	297.6 ± 4.9*	1800.9 ± 14.1*	611.1 ± 20.5*
3-10 kDa	553.4 ± 2.3[b]	632.2 ± 10.3*	1841.1 ± 12.1*	496.5 ± 6.1*
>10 kDa	227.1 ± 4.3[c]	540.8 ± 8.7*	1480.9 ± 13.9*	802.8 ± 20.9*
Positive control	-	33.9 ± 0.9 (EDTA disodium)	121.3 ± 0.2 (EDTA disodium)	51.2 ± 0.5 (Reduced glutathione)

Data are presented as mean ± SEM (n = 3). For peptide content data, values followed by different superscripts (a-c) are significantly different (p < 0.05) as determined by Fisher's LSD test. For EC_{50} data, asterisks (*) indicate mean values that are significantly different (p < 0.05) from that of the positive control, as determined by Student's t-test

When BSA was treated with the Fenton's reagent without any added hydrolysates or fractions, the remaining amount of BSA detected was negligible (Fig 2A, lane 2). However, supplementation of hydrolysates and ultrafiltration fractions significantly increased the amount of BSA that remained after exposure to Fenton's reagent (Fig 2B). 3AH supplementation at 20 mg/mL resulted in higher BSA band intensity (56.4 %) compared with 3PH (47.1 %) and 3TH (40.8 %). When the three ultrafiltration fractions were compared, addition of < 3 kDa fraction to BSA resulted in the highest band intensity (56.3 %). Notably, addition of 3AH and < 3 kDa fraction to BSA both resulted in band

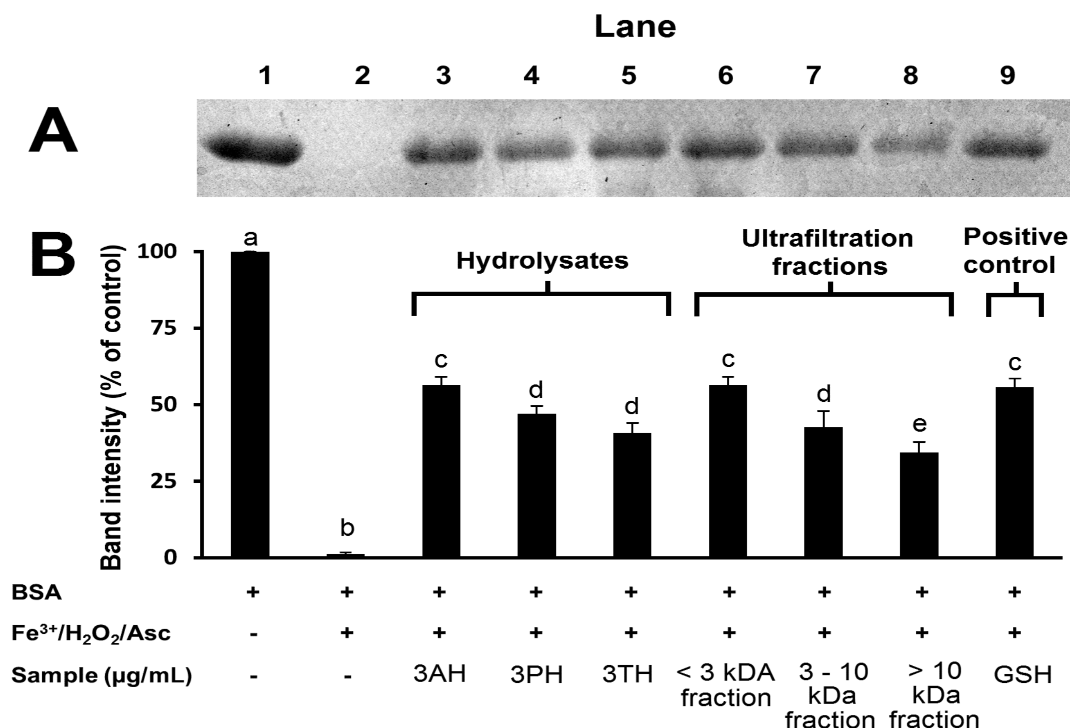

Fig 2: A typical gel image of BSA treated with Fenton's reagent (Fe^{3+}/H_2O_2/ascorbic acid) in the presence and absence of hydrolysates and ultrafiltration fractions (A) as well as densitometric analysis of the corresponding BSA bands (B). Each bar represents mean ± SEM (n=3). Mean values denoted by different superscript letters are significantly different ($p < 0.05$) as determined by Fisher's LSD test. GSH, reduced glutathione

intensities comparable to that of BSA supplemented with reduced glutathione.

DISCUSSION

Different proteinaceous raw materials, when hydrolysed, are known to yield hydrolysates with different bioactive and nutritional values [2]. The antioxidant properties of protein hydrolysates derived from numerous bony marine fishes have been reported [2,3]. However, studies on the antioxidant potential of protein hydrolysates prepared from edible, cartilaginous marine fishes are uncommon. In this study, we demonstrated for the first time the antioxidant capacity of protein hydrolysates and membrane ultrafiltration fractions prepared from the blue-spotted stingray. Among the three proteases used, alcalase hydrolysis stood out as the best for the production of protein hydrolysate exhibiting antioxidant activity. Hydrolysis with alcalase, unlike that with papain and trypsin, consistently enhanced the iron chelating, copper chelating and ABTS radical scavenging activities of the resultant 3-h hydrolysate. 3AH also exhibited the strongest metal chelating and ABTS radical scavenging activities among the 3-h hydrolysates. Our results concur with previous observations of the ability of alcalase hydrolysates of fish proteins to chelate pro-oxidant metal ions and scavenge free radicals [3]. Furthermore, among the three protease treatments, hydrolysis with alcalase led to the highest peptide content in the 3 h hydrolysate. This finding agrees with the proposition that alcalase and other microbial proteases are relatively effective in the production of FPH, which explains their wider uses compared with plant proteases (e.g. papain) and animal proteases (eg. trypsin) in hydrolysate production [2].

Considering that 3AH had the most promising metal chelating and ABTS radical scavenging activities, we fractionated 3AH to yield three peptidic fractions by using the membrane ultrafiltration technique. The 3 -10 kDa fraction had the highest peptide content, implying that a large proportion of the peptides in 3AH were likely between 3 kDa and 10 kDa in molecular size. All three fractions, however, showed lower iron chelating activity than 3AH. This suggests that the iron chelating activity detected in 3AH may have resulted from additive or synergistic actions of the three peptide fractions. On the other hand, the copper chelating activity of all three ultrafiltration fractions surpassed that of 3AH. This suggests that the three peptide fractions may have exerted antagonistic effects among themselves in 3AH. Both < 3 kDa and 3 -

10 kDa fractions showed higher ABTS radical scavenging activity than 3AH, with 3 -10 kDa fraction being the stronger of the two. Hence, the 3 -10 kDa fraction was possibly a key contributor to the ABTS radical scavenging activity detected in 3AH. In general, the lowest molecular weight hydrolysate fraction (< 3 kDA) was associated with the highest iron chelating but not with the highest copper chelating and ABTS radical scavenging activities. This is similar to the findings on the ultrafiltration fractions of silver carp hydrolysate prepared with alcalase treatment [13]. The study found that the lowest molecular weight hydrolysate fraction may or may not have the highest antioxidant activity, depending on the type of antioxidant parameters tested.

We have used the ABTS radical scavenging assay to compare the antioxidant activities of the protein hydrolysates and ultrafiltration fractions. The assay is widely used for antioxidant screening owing to its rapid and simple nature. ABTS, nevertheless, are not biological-relevant free radicals. Thus, we further characterised the antioxidant potential of the 3 h hydrolysates and ultrafiltration fractions by evaluating their hydroxyl radical scavenging activity and their protective effects against oxidative protein damage. Hydroxyl radicals are the most reactive of all ROS, capable of reacting with and damaging most biomolecules in living cells [14]. Proteins are the major targets of ROS in cells not only due to their high abundance as well as their active participation in most cellular processes. Oxidative protein damage is associated with aging and human pathologies [15].

The < 3 kDa fraction was as potent as reduced glutathione in quenching hydroxyl radicals and in protecting against oxidative protein damage. The results suggest that peptides of less than 3 kDa in size may be the primary contributor to the hydroxyl radical scavenging and protein-protective effects observed in 3AH. Notably, the findings on the potency of the < 3 kDa fraction also support previous observations that the antioxidant effects of FPH are often attributed to peptides of less than 3 kDa in length [2,3]. Furthermore, antioxidant potential of FPH is also influenced by their amino acid composition and sequence [2]. Thus future research is warranted to investigate the two parameters in the < 3 kDa fraction to better understand the molecular basis for its promising antioxidant activity.

CONCLUSION

Alcalase is more effective than papain and trypsin when used to produce antioxidative protein hydrolysate from blue-spotted stingray. Hydrolysis with alcalase enhances the peptide content as well as metal chelating and ABTS radical scavenging activities of the hydrolysate. Importantly, the < 3 kDa peptide fraction is as potent as reduced glutathione in scavenging hydroxyl radicals and in protecting proteins from oxidative damage. Our results suggest that alcalase hydrolysate and specifically its < 3 kDa fraction have promising potential for future applications in antioxidant therapy and health food formulation.

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