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Graphite Furnace Atomic Absorption Spectrophotometric Determination of Trace Horseradish Peroxidase Using Nanosilver

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In pH 4.2 HAc-NaAc buffer solution, horseradish peroxidase (HRP) catalyzed H_2O_2 oxidation of nanosilver to form Ag^+. After centrifugation, Ag^+ in the supernatant can be measured by graphite furnace atomic absorption spectrophotometry (GFAAS) at the silver absorption wavelength of 328.1 nm. When HRP concentration increased, the Ag^+ concentration in the supernatant increased, and the absorption value enhanced. The HRP concentration in the range of 0.84-50 ng·mL^{-1} was linear to the enhanced absorption value (ΔA), with a regression equation of $\Delta A = 0.012C + 0.11$, correlation coefficient of 0.9988, and detection limit of 0.41 ng·mL^{-1} HRP. The proposed GFAAS method was used to detect HRP in waste water samples, with satisfactory results.

Key Words : Horseradish peroxidase, Nanosilver, Graphite furnace atomic absorption spectrophotometry

Introduction

Metal nanoparticles show unique optical and electrical properties owing to their surface, volume, quantum size and macroscopic quantum tunnel effect, and have been used in analytical chemistry.[1-3] Nanosilver is a kind of the most affordable precious metal functional materials with high surface activity, and has been widely used as catalyst, antibacterial material, deodorant and ultraviolet radiation absorbent, and biosensor. Recently, it has been applied in biochemical analysis due to its novel spectral characteristics.[4,5] Horseradish peroxidase (HRP) is a very important protease and can catalytically oxidize some inorganic and organic compounds, that utilized widely in the fields of environmental science, clinical chemistry, food industry, analytical chemistry and so on.[6-12] Thus, its detection is very important. Presently, the main assays for detection of HRP activity including voltammetry,[12] spectrophotometry,[13-15] fluorescence spectrometry,[16] chemiluminescence,[17] flow injection analysis,[18] resonance scattering methods.[19] The reagent of chromotrope 2R showed a voltammetric peak, and the peak decreased after adding HRP and H_2O_2. Accordingly, 4.0×10^{-8} - 4.0×10^{-6} g·mL^{-1} HRP can be determined by voltammetry.[12] Using the system of quaternary ammonium-H_2O_2-HRP, as low as 3×10^{-10} g·mL^{-1} HRP can be determined spectrophotometrically by enzyme-linked immunoassay.[13] However, some of above method were not sensitive, some were not selective, and some method operations are complex. Atomic absorption spectrometry (AAS), especially graphite furnace atomic absorption spectrometry (GFAAS), is of high sensitivity and selectivity to determine metal elements,[20,21] and recently applied in the indirect analysis of some organic compounds.[22] Up to date, there is no report about GFAAS method for HRP using enzyme catalytic oxidation nanosilver reaction. Thus, establishing a highly selective and sensitive assay for HRP has a great significance. In this article, based on H_2O_2 oxidizing nanosilver to form Ag^+ in the presence of HRP catalyst, and measuring the Ag^+ in the supernatant by GFAAS, a highly sensitive and selective, and simple GFAAS assay has been proposed to detect HRP in water samples.

Experimental

Apparatus and Reagents. Model TAS-990 atomic absorption spectrometer (Beijing Puxi General Instrumental Company, China) was used to record silver absorption value with a measurement wavelength of 328.1 nm, hollow cathode lamp current of 2.0 mA, spectral band width of 0.4 nm, measurement mode of the integration mode, measurement time of 3 s, filter coefficients of 0.10. Graphite furnace temperature program for silver was listed as in the Table 1. A model UV-1901 UV-visible spectrophotometer (Beijing Purkinje General Instrument Limited Co., China), a model XT-9900 intelligent microwave digestion instrument (Shanghai Xintuo Analytical Instruments Limited Co., China), a model RETBC1001 heating magnetic stirrer (IKA Group, Germany), a model Sigma 3K30 high speed freezing centrifuge (Sigma Company, Germany), a model SK1200H ultrasonic cleaner (Shanghai Kudos Ultrasonic Instrument Limited Co.,

Table 1. Parameters for the graphite furnace temperature program

Step	Temperature (°C)	Ramp time (s)	Hold time (s)	Ar gas flow rate (mL/min)
Drying	80-100	15	10	450
Pyrolysis	500	10	8	450
Atomisation	1800	0	3	0
Clearing	1900	1	2	450

China), a model DK-8B thermostat (Shanghai Jing Hong Laboratory Instrument Limited Co., China), and a model PT-10 pH meter (Sartorius, Germany) were used.

A 2.0×10^{-3} mol·L^{-1} $AgNO_3$ solution and 1% sodium citrate were prepared. 2.0×10^{-3} mol·L^{-1} sodium borohydride ($NaBH_4$) were prepared freshly. Horseradish peroxidase (3300 U·mg^{-1}) was purchased from Huamei Biotechnology Company, China, and was used to prepare a 10 μg·mL^{-1} solution with water. A 0.2 mol·L^{-1} acetic acid (HAc) and 0.2 mol·L^{-1} sodium acetate (NaAc) were used to prepare pH 3.8-4.8 buffer solution (HAc-NaAc) according to a certain volume ratio. A 3.96×10^{-2} mol·L^{-1} hydrogen peroxide (H_2O_2) stock solution was standardized using potassium permanganate ($KMnO_4$) standard solution. All the reagents were analytical grade and the used water was doubly distilled.

Sodium Borohydride Reducing Procedure for Preparation of Nanosilver. Nanosilver was prepared by the following procedure. Firstly, 1.0 mL, 2.0 mL, 3.0 mL, 4.0 mL, 6.0 mL, 9.0 mL, 10.0 mL, 12.0 mL, 13.0 mL, 18.0 mL, 21.0 mL of 2.0×10^{-3} mol·L^{-1} $NaBH_4$ were added to small beakers respectively. Then, 3.0 mL of 2.0×10^{-3} mol·L^{-1} $AgNO_3$ solution was dropped to beakers and stirred for 30 min, The color changed to pale yellow, which indicated that the nanosilver particles formed. Finally, these solutions were transferred to 25 mL flasks, diluted to 25 mL. Then 1.0 mL of solution was diluted to 3.0 mL, the absorption value was measured by ultraviolet-visible spectrophotometer (Fig. 1). Results showed that, with addition of $NaBH_4$, nanosilver concentration increased, and the absorption value enhanced. When the volume was greater than 10 mL, the absorption value changed little, which indicated that Ag^+ had been reduced completely, and reducing agent had little effect. So, a 12.0 mL $NaBH_4$ was used to reduce 3.0 mL of 2.0×10^{-3} mol·L^{-1} $AgNO_3$. The concentration, calculated as Ag, was 2.4×10^{-4} mol·L^{-1}, the average size was 5 nm, and the solution kept in dark place and stored at 4 °C.

Sodium Citrate Microwave Reducing Procedure for Preparation of Nanosilver: A 1.2 mL 2.0×10^{-3} mol·L^{-1} $AgNO_3$ and 1.0 mL 1% sodium citrate were added in a digestion tank, mixed well and irradiated 4 min at 0.9 MPa by microwave instrument. The mixture was diluted to 10 mL, and was centrifuged at 9000 rpm for 15 min to remove the supernatant. Then 10 mL water was added in the tube, and the particles were dispersed by ultrasounic wave for 15 min, centrifuged twice by the same operation, and diluted to 10 mL. The concentration, calculated as Ag, was 2.4×10^{-4} mol·L^{-1},[23] with average size of 10 nm.

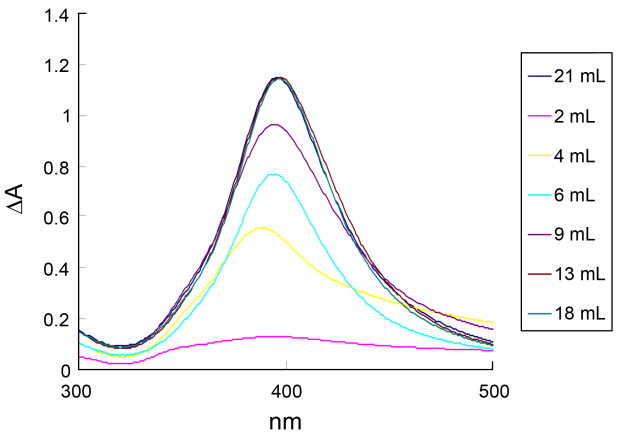

Figure 1. UV-vis absorption spectra of nanosilver.

Sodium Citrate Heating Reducing Procedure for Preparation of Nanosilver. A 12.0 mL 2.0×10^{-3} mol·L^{-1} $AgNO_3$ solution and 100 mL water were added to a beaker. The solution was heated in water bath at 90 °C. Then 5 mL of 1% sodium citrate solution was added slowly under stirring (120 rpm) and the solution was kept at 90 °C for 60 min. Then it was transferred into a 100 mL volumetric flask, and diluted to the mark. The concentration, calculated as Ag, was 2.4×10^{-4} mol·L^{-1}, with an average size of 15 nm. Finally, the solution was centrifuged and purified as same as above operation, and was kept in dark place and stored at 4 °C.

Procedure for Assay of HRP. A 100 μL of pH 4.2 HAc-NaAc buffer solution, 40 μL 3.96×10^{-2} mol·L^{-1} H_2O_2 solution, 1.0 mL 2.4×10^{-4} mol·L^{-1} silver colloid, and a certain amount of HRP were added to a 5 mL graduated tube successively. The mixture was diluted to 3.0 mL and reacted for 6 min at 30 °C. Then, the mixture was centrifuged at 24000 rpm for 20 min to obtain the supernatant. A 100 μL of the supernatant was added to a 5 mL graduated tube, and diluted to 2.0 mL. The absorption value at 328.1 nm (A) was recorded by atomic absorption spectrometry. The A_b value of the blank solution without HRP was also measured. The value of $\Delta A = A - A_b$ was calculated.

Results and Discussion

Principle. Nanosilver particles are oxidized difficultly by H_2O_2 in weak acid condition. Upon addition of HRP catalyst, it can catalyze H_2O_2 to generate hydroxyl radical, which has strong oxidation, and can oxidize nanosilver particles to form silver ions. According to our results and the references,[19] the main reactions are as follows,

$$HRP + H_2O_2 \xrightarrow{\text{Catalyzed}} \bullet OH \quad (1)$$

$$(Ag)_n + \bullet OH \longrightarrow Ag^+ + OH^- \quad (2)$$

According to above reactions, we know,

$$C_{\bullet OH} \propto C_{HRP} \quad (3)$$

$$C_{Ag^+} \propto C_{\bullet OH} \quad (4)$$

Where $C_{\bullet OH}$, C_{HRP} and C_{Ag^+} stand for the concentration of hydroxyl radicals, HRP and Ag^+ respectively. Based on AAS principle, we have known,

$$A \propto C_{Ag^+} \quad (5)$$

According to formula (3)-(5), we get,

$$A \propto C_{Ag^+} \propto C_{\bullet OH} \propto C_{HRP} \quad (6)$$

The formula (6) indicated that when HRP concentration

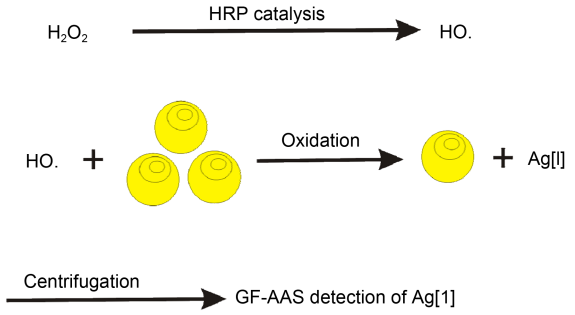

Figure 2. Principle of GFAAS assay of HRP.

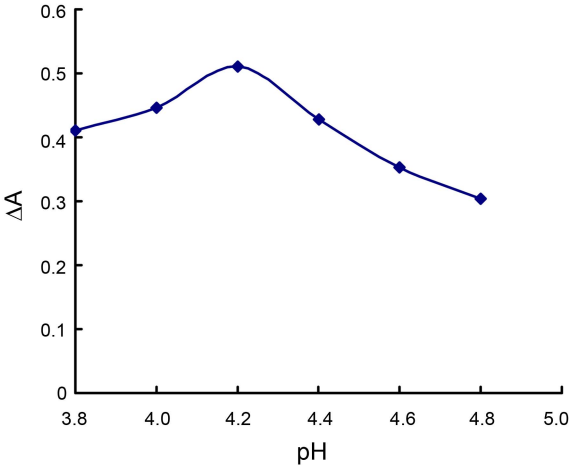

Figure 3. Effect of pH.
26.4 μmol·L^{-1} H_2O_2 - 40 μmol·L^{-1} nanosilver –33.3 ng·mL^{-1} HRP.

increased the concentration of hydroxyl radical increased, the amount of oxidized nanosilver particles increased, the Ag^+ concentration in the supernatant increased, and the absorption value increased linearly. Based on these grounds, a rapid, sensitive and selective GFAAS assay for HRP was established (Fig. 2).

Influence of Adding Reagents Sequence. The adding sequence of buffer solution, H_2O_2, HRP, and nanosilver was studied. As Table 2 showed that the ΔA value was minimal, when the adding sequence was buffer solution + nanosilver + H_2O_2 + HRP was used. The ΔA value was maximal when adding sequence of buffer solution + H_2O_2 + nanosilver + HRP was selected for use.

Effect of pH. Effect of pH HAc-NaAc buffer solution in the range of 3.8-4.8 was considered. Figure 3 showed that when the pH value was 4.2, the most hydroxyl radicals formed, and the ΔA was maximal. With addition volume of buffer solution increased, the absorption value increased. But the Ac^- concentration was higher than 10 mmol·L^{-1}, the blank absorption increased. Thus, a 0.1 mL pH 4.2 HAc-NaAc buffer solution with the concentration of 6.67 μmol·L^{-1} Ac^- was used.

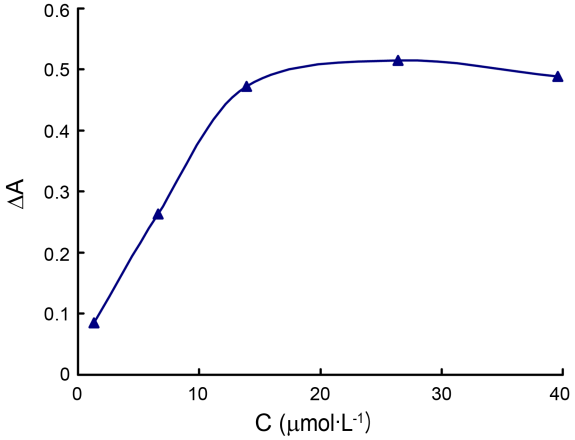

Figure 4. Effect of H_2O_2 concentration.
pH 4.2 HAc-NaAc-40 μmol·L^{-1} nanosilver –33.3 ng·mL^{-1} HRP.

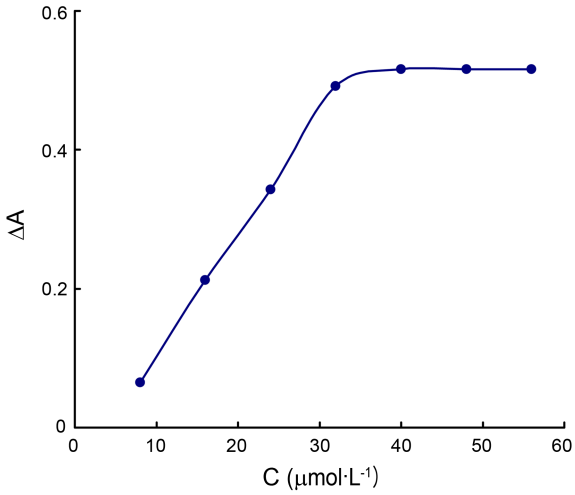

Figure 5. Effect of nanosilver concentration.
pH 4.2 HAc-NaAc -26.4 μmol·L^{-1} H_2O_2 - 40 μmol·L^{-1} nanosilver - 33.3 ng·mL^{-1} HRP.

Effect of H_2O_2 Concentration. Effect of H_2O_2 concentration in the range of 1.32-39.6 μmol·L^{-1} on the ΔA was studied. Results showed that when the H_2O_2 concentration was in the range of 1.32-26.4 μmol·L^{-1}, the formed hydroxyl radical increased and the absorption value increased with the H_2O_2 concentration. When the H_2O_2 concentration was 26.4 μmol·L^{-1}, the formed hydroxyl radical was most, and the ΔA was maximal (Fig. 4). Thus, a concentration of 26.4 μmol·L^{-1} H_2O_2 was used.

Effect of Nanosilver Concentration. The effect of nanosilver concentration on the ΔA was considered. Results showed in Figure 5. When nanosilver concentration increased,

Table 2. The influence of adding reagents sequence on ΔA

Sequence	buffer solution + nanosilver + H_2O_2 + HRP	buffer solution + H_2O_2 + HRP + nanosilver	buffer solution + H_2O_2 + nanosilver + HRP	buffer solution + HRP + nanosilver + H_2O_2
ΔA	0.486	0.502	0.521	0.495

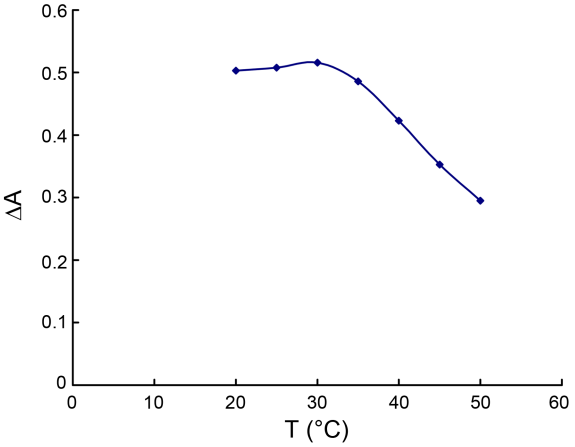

Figure 6. Effect of temperature.
pH 4.2 HAc-NaAc - 26.4 μmol·L^{-1} H_2O_2 - 40 μmol·L^{-1} silver nanoparticles - 33.3 ng·mL^{-1} HRP.

the oxidized nanosilver increased, and the Δ*A* value enhanced greatly. When the nanosilver concentration was 40 μmol·L^{-1}, the Δ*A* value was maximal. The nanosilver increased further, the absorption value changed little, it indicated that the amount of nanosilver was enough, so a 40 μmol·L^{-1} nanosilver was used. In this assay, because the absorption value of Ag^+ concentration in the supernatant was measured, the relationship between Ag^+ concentration and absorption value was examined. Results showed the absorption value was linear to Ag^+ concentration in the range of 2.0×10^{-8} - 1.2×10^{-6} mol·L^{-1}, with a regression equation of $A = 0.6886\ C_{Ag} + 0.0595$, and relative coefficient of 0.9952, thus the supernatant was diluted before measured.

Effects of Reaction Temperature and Reaction Time. Enzyme is a kind of protein, and the protein will deteriorate and lose their activity if the temperature is too high, so the temperature on the catalytic reaction has a great influence. In this article, the effect of temperature (20-50 °C) on the Δ*A* value was examined. Figure 6 showed that the Δ*A* reached maximum at 30°C. The Δ*A* values decreased greatly as the reaction temperature was higher than 35 °C. When the reaction time was 6 min, the Δ*A* value was maximal and kept stable within 40 min. Thus, reaction time 6 min at 30 °C was chosen for use.

Effect of Centrifugation Speed and Time. The effect of centrifugation speed and time on the Δ*A* value was studied, respectively. Results showed that the centrifugation speed was in the range of 16000-26000 rpm, the Δ*A* increased (Fig. 7). When the centrifugation speed was higher than

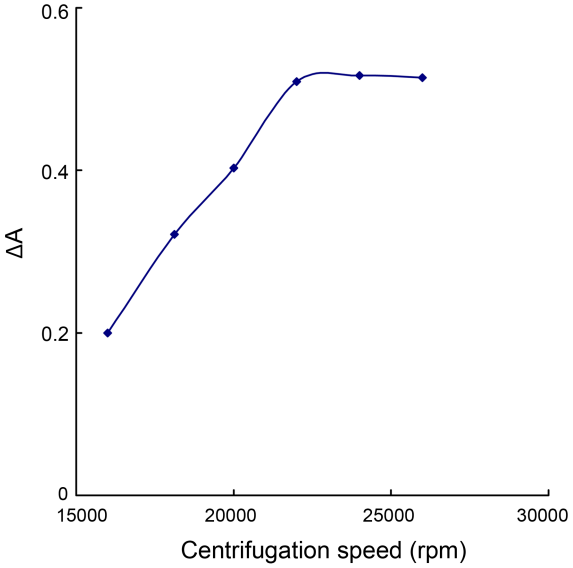

Figure 7. Effect of centrifugation speed on Δ*A*.
pH 4.2 HAc-NaAc -26.4 μmol·L^{-1} H_2O_2 - 40 μmol·L^{-1} silver nanoparticles - 33.3 ng· mL^{-1} HRP.

24000 rpm (centrifugation force was 48942RCF), the Δ*A* was reached maximum. When the centrifugation time was within 20 min, the Δ*A* value reached maximum and stabilized. Thus, 24000 rpm for 20 min was chosen.

Working Curve. According to the procedure, the Δ*A* value for different HRP concentrations *C* (ng·mL^{-1}) was recorded. The analytical features of the nanosilver on the Δ*A* were showed in Table 3. The nanosilver prepared by the sodium borohydride reduction procedure had a low detection limit and wide detection range. For this nanosilver,

Table 4. Selectivity of the method (33.3 ng·mL^{-1} HRP)

Coexisting Substance	Tolerance [CS]/ [HRP]	Relative error (%)	Coexisting Substance	Tolerance [CS]/ [HRP]	Relative error (%)
Mg^{2+}	210	+5.1	Mn^{2+}	160	–5.8
Pb^{2+}	200	+5.3	Cr^{3+}	90	+3.6
Al^{3+}	80	–6.7	Zn^{2+}	220	+6.3
Fe^{3+}	90	+6.1	Ni^{2+}	210	+5.3
Co^{2+}	300	–3.7	Hg^{2+}	150	–4.7
Ca^{2+}	250	+5.8	Cl^-	150	–5.5
K^+	400	–7.5	NO_3^-	300	–6.1
NH_4^+	380	+4.5	CO_3^{2-}	240	+4.4
Cd^{3+}	180	+6.5	SO_4^{2-}	300	–3.6

Table 3. Analytical features of the different nanosilver systems

Nanosilver size	Regression equation	Linear range (ng·mL^{-1})	Detection limits (ng·mL^{-1})	Correlation coefficient
5 nm[a]	$\Delta A = 0.012\ C + 0.11$	0.84-50.0	0.41	0.9988
10 nm[b]	$\Delta A = 0.0062\ C + 0.23$	2.08-33.3	1.27	0.9983
15 nm[c]	$\Delta A = 0.0051\ C + 0.01$	0.84-33.3	0.60	0.9968

[a]Nanosilver prepared by the sodium borohydride reducing procedure reduction. [b]Nanosilver prepared by the sodium citrate microwave reducing procedure reduction. [c]Nanosilver prepared sodium citrate heating reducing procedure reduction.

Table 5. Analytical results for HRP concentration and recovery in samples

Sample	Single value ($ng \cdot mL^{-1}$)	Average ($ng \cdot mL^{-1}$)	RSD (%)	Added ($ng \cdot mL^{-1}$)	Found ($ng \cdot mL^{-1}$)	Recovery %
1	8.42, 8.61, 8.72, 8.55, 8.67	8.59	1.4	16.67	16.82	100.9
2	2.85, 2.72, 2.79, 2.63, 2.78	2.75	3.0	16.67	15.73	94.4
3	14.6, 15.7, 15.0, 14.8, 16.1	15.24	4.2	16.67	16.26	97.5

as the size reduced (about 5 nm), the surface enlarged, so the oxidation of nanosilver by H_2O_2 enhanced, the slope value increased, and a lower detection limit can be achieved. So this nanosilver was chosen for determination of HRP.

Influence of Foreign Substances. According to the procedure, the influence of foreign substance on the determination of 33.3 $ng \cdot mL^{-1}$ HRP was examined, with a relative error of ± 10%. Tolerance of weight ratio of coexistence ions was shown in Table 4. Some common ions did not significantly interfere with the HRP determination. Thus, this assay has good selectivity.

Determination of Sample. Appropriate waste water was filtered and determined according to the procedure. The results were showed in Table 5. A known amount of HRP was added to the samples to determine recovery, and the recovery was 94.4-100.9%.

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