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Test-Strip-Type Salivary Amylase Activity Monitor and Its Evaluation

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The authors have been focusing on the activity of α-amylase in saliva (salivary amylase) with the aim of developing a simple quantitative measurement technique for monitor human stress. However, the measurement of enzymatic activity required a sufficiently large volume of substrate and the realization of quantification also required some mechanism to control reaction time. Moreover, a measurement method using saliva samples should have the merits of simplicity, the ability to yield instant results and the potential to be used any time. Based on this concept, a salivary amylase activity monitor consisting of the test strip, a salivary transcription device and an optical analyzer was fabricated. The calibration curve for the salivary amylase activity monitor obtained an R^2 value of 0.72. The monitor could be used for the analysis of salivary amylase activity. In order to evaluate the monitor, salivary amylase activity was measured using a videotape of corneal transplant surgery as a mental stressor. A significant difference between salivary amylase activity was recognized between the prestress, midstress and poststress periods, and it was confirmed that increasing or decreasing human stress level could be perceived as increases and decreases in salivary amylase activity.

1. Introduction

The concentrations of glucocorticoid, a type of steroid hormone, and catecholamine, a neurotransmitter, are often used as an index to evaluate the level of human stress.[1] In particular, various reports have been published covering the use of cortisol, a type of

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glucocorticoid, because it is considered as a salient index.[2–4] However, since its concentration in blood or saliva is very low, the analysis requires not only specific large-scale equipment, such as HPLC (high-performance liquid chromatography) or EIA (enzyme immunoassay) but the analysis also takes a long time to complete.

Therefore, the authors have been focusing on the activity of α-amylase in saliva (salivary amylase) with the aim of developing a simple quantitative measurement technique to monitor human stress. Since salivary amylase is a digestive enzyme and its secretion is innervated by the sympathetic nervous system, changes in its activity that are related to levels of stress can be expected.[5–7] Previous studies have investigated using the Kraepelin Test as a stressor and confirmed that there are significant changes in salivary amylase activity caused by stress.[8] However, the measurement of enzymatic activity requires a sufficiently large volume of substrate and the realization of quantification also requires some mechanism to control reaction time. Moreover, a measurement method using saliva samples should have the merits of simplicity, the ability to yield instant results and the potential to be used any time.

Based on this concept, the authors fabricated a prototype portable salivary amylase activity monitor to analyze enzymatic activity. This monitor consisted of

1. a disposable test strip that can collect saliva in μl quantities,
2. a saliva transcription device that can control amylase reaction time,
3. an optical analyzer that measures the color density of the test strip.

The test strip consisted of a collection strip to quantify saliva and a reagent strip to measure salivary amylase activity by determining color density.

In this report, we explain the structure and principles of the test strip and saliva transcription device. Then we describe the characteristics of the saliva transcription device and the evaluation results for the calibration curve of the amylase activity monitor. Finally, we report the results of an experiment on salivary amylase activity using healthy young adults who watched a videotape of corneal transplant surgery as a mental stressor.

2. Materials and Methods

2.1 *Salivary amylase activity monitor*

We fabricated a salivary amylase activity monitor that consisted of a test strip, a saliva transcription device and an optical analyzer (Fig. 1). The test strip consisted of a collection strip for collecting saliva and a reagent strip for measuring the salivary amylase activity (*AMY*). Both are disposable. A nonwoven material tape (7×5×0.15 mm^3, drying volume of 5.25 μl) was bonded to the tip of a plastic plate (60×5×0.3 mm^3) using double-sided tape, and this tip was used as the collection strip. Next, an amylase activity testing tape was fabricated to measure salivary amylase activity. 0.13 mmol (86 mg) of Gal-G2-CNP and 7.0 mmol (680 mg) of potassium thiocyanate (KSCN) were dissolved in 1 ml of Good's buffer containing 2-morpholinoethanesulfonic acid (MES, CAS No.4432-31-9) as the main component. A filter paper was then soaked in Good's buffer, and was dried in a drying oven (DK-400, Yamato Scientific Co., Ltd., Japan) at 30°C for about 30 min. Gal-G2-CNP (molecular weight: 659.98) is a new chromogen that has recently been used for the measurement of amylase activity in blood,[9] and it acts as a substrate of α-amylase. KSCN was added to Good's buffer as a stabilizer of Gal-G2-CNP. The manufactured

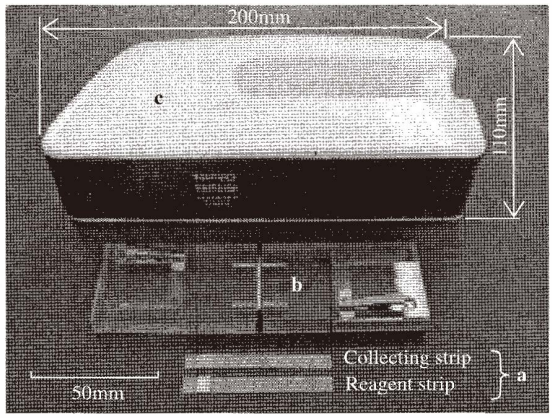

Fig. 1. External view of the test-strip-type salivary amylase activity monitor fabricated. a, test-strip; b, saliva transcription device; and c, optical analyzer.

amylase activity testing tape (6×5×0.25 mm^3, drying volume of 7.5 μl) was bonded to the tip of a plastic plate (60×5×0.3 mm^3) using double-sided tape, and this tip was used as the reagent strip. When saliva was applied onto the amylase activity testing tape, CNP was liberated and it took on a yellow color.

$$\text{Gal-G2-CNP} \xrightarrow{\alpha\text{-amylase}} \text{Gal-G2} + \text{CNP} \qquad (\text{white} \rightarrow \text{yellow}) \tag{1}$$

This enzyme reaction would continue until all the substrate was consumed. In order to determine enzymatic activity quantitatively, a control mechanism of reaction time was necessary. Then, the saliva transcription device was fabricated. The collection strip and reagent strip were set in the saliva transcription device at the start of measurement. Next, a cotton roll (ϕ 8 mm×25 mm) was set under the tongue, and left in place for between 30 s to 1 min to collect whole saliva. Immediately after, the cotton roll was condensed using a medical syringe (γ-ray-sterilized, NIPRO Co., Japan) and the saliva was applied onto the collection strip. When the saliva transcription device was bent, the collection and reagent strips were placed in contact with each other. This time was set as the initial time. The strips were placed in this condition for about 20 s and a certain quantity of the saliva was transferred from the collection strip to the reagent strip (Fig. 2(a)). Immediately after this, the reagent strip was inserted into the optical analyzer (200×110×50 mm, 760g), which had a three-light-source-type light-emitting diode (LED, CZ-10, KEYENCE Co., Japan, Fig. 2(b)). 30 s after the initial time, the coloring concentration was analyzed using the optical analyzer (Fig. 2(c)). The result was displayed from 0 to 999 in proportion to salivary amylase activity (Fig. 2(d)).

2.2 *Monitor characteristics*

To verify the measurement accuracy of the amylase activity testing tape, a calibration curve was measured. The saliva used for this evaluation was collected from seven healthy

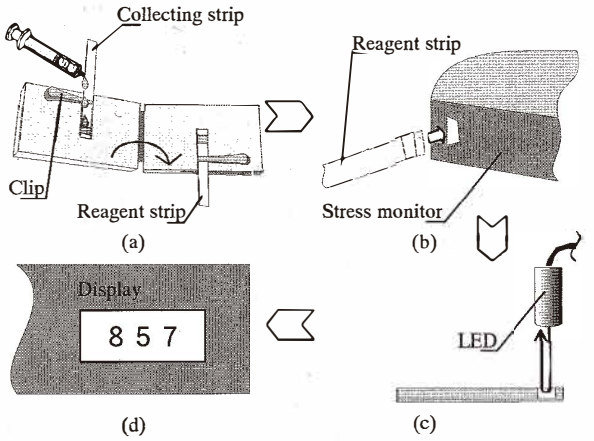

Fig. 2. Measurement protocol of the salivary amylase activity monitor (LED: light-emitting diode).

young adults who had no oral diseases (four male and three female, 21–24 yr). The aim of the experiment was explained to the subjects and consent was obtained after confirmation that they fully understood the experiment. A cotton roll was set under each subjects tongue. The cotton roll was condensed using a syringe and whole saliva was collected in under nonstimulation conditions. The saliva samples were diluted to 75%, 50% and 25% using 1% bovine serum albumin (BSA) solution. 5 μl of the saliva sample was measured using a micropipette (0.1 μl resolution, 1.5% of the maximum error) and was dropped on the reagent strip directly. 30 s after the initial time, coloring concentration was analyzed using the optical analyzer. That is, the saliva transcription device was not used in this stage to measure the characteristics of the amylase activity testing tape only. Simultaneously, the activity of the collected saliva samples was measured using an enzymatic method reagent (Espa AMY-FS, Nipro Co., Japan)[10] and a clinical automatic analyzer (Miracle Ace 919, Nipro Co., Japan). This result was used as the absolute value of α-amylase activity. One unit activity (U) per mass of enzyme was defined as the activity that produces 1 μmol of the reducing sugar, maltose, per minute.

Next, a total calibration curve of the salivary amylase activity monitor was measured under conditions using the saliva transcription device. A cotton roll was condensed using a syringe and whole saliva was collected without being measured quantitatively; then the saliva sample was dropped on the collection strip. Saliva was transferred from the collection strip to the reagent strip by the saliva transcription device. 30 s after the initial time, coloring concentration was measured by the optical analyzer. At the same time, the absolute value of salivary amylase activity was determined using a clinical automatic analyzer. The volume of the saliva sample was estimated from the mass change of the reagent strip by setting the specific gravity to be 1. An electronic analytical balance (0.01 mg of optimum sensitivity, HM-202, A&D Co. Ltd, Japan) was used for this measurement where 0.1 mg corresponded to 0.1 μl.

2.3 *Stress evaluation*

The subjects were shown a videotape of corneal transplant surgery (Discovery Communications, Inc., USA) as a mental stressor while their salivary amylase activity was analyzed using the salivary amylase activity monitor fabricated. Since this videotape was produced for education in surgical procedures, scenes that might normally be expected to have an emotional impact were not edited out. The subjects were six healthy young adults without any oral disease and aged between 21 and 23 years old, consisting of two females (A and B) and four males (C, D, E and F). The aim of the experiment was explained to the subjects and consent was obtained after confirmation that they fully understood the experiment. In order not to affect the subjects' stress, detailed information about the contents of the videotape was not revealed beforehand. The experiments were conducted in the morning, two hours or more after breakfast.

Firstly, the subjects were kept in a sitting position for 30 min to ease their stress. Prior to the start of the experiment, saliva secreted in their mouths was removed using a dental cotton roll, and the time was set to 0 min. They were further maintained in a sitting position for 8 min and saliva was collected at 1-min intervals under the prestress condition. The method of collecting saliva was the same as mentioned in 2.1. A cotton roll was set under the tongue in order to collect whole saliva without any stimulation. After this, the subjects viewed the videotape in a sitting position for 10 min. While the videotape was running, midstress saliva was collected 5 min and 10 min after the tape started. Finally, the subjects were further maintained in a sitting position for 8 min while poststress saliva was again collected at 1-min intervals. Throughout the test, the time taken for each saliva collection operation was maintained at 1 min. The mean salivary amylase activity for the 8 min (n=8) before loading the stress was calculated and defined as the prestress salivary amylase activity. Also, the mean salivary amylase activity at 5 min and 10 min after the start of the videotape viewing was defined as the midstress salivary amylase activity. The mean salivary amylase activity during the 8 min after stress loading was defined as the poststress salivary amylase activity.

Moreover, to study the effect of the secretion rate of saliva, the volume per minute (ml/min) of salivary secretion was also calculated. Finally, the correlation between the prestress, midstress and poststress salivary amylase activities was statistically evaluated using the Wilcoxon test method. Proprietary statistical software (Stat View, SAS Institute Inc.) was used for the calculation.

3. Results

3.1 *Monitor characteristics*

The salivary amylase activity of the whole saliva collected from the seven subjects ranged from 57.6 to 423.5 kU/L. By diluting the sample with 1% BSA solution to 75%, 50% and 25% respectively, 28 samples were obtained in which the salivary amylase activity ranged from 14.4 to 423.5 kU/L. These saliva samples were analyzed to compare the results between using the amylase activity testing tape and the clinical automatic analyzer. The results showed that the calibration curve had $y = 14.99e^{0.0116x}$ with an R^2 value of 0.88 (Fig. 3). When salivary amylase activity was higher than 200 kU/L, the change in

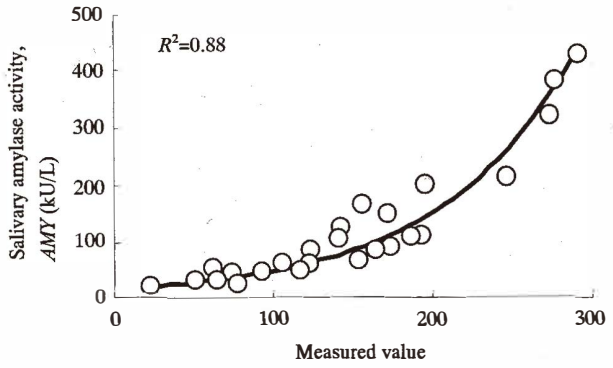

Fig. 3. Calibration curve of the amylase activity testing tape measured by the optical analyzer (no use of saliva transcription device).

the coloring concentration of the testing tape decrease and showed a tendency towards saturation. Also, when the reaction time was intentionally varied from 30 s plus or minus 20% (6 s), the measured result varied by up to 30%.

Next the saliva samples of the seven subjects were transcribed using the saliva transcription device. Individual differences between the fluidic characteristics of the saliva were observed, but the volumes were too small to measure viscosity coefficient. The volume of the transcribed saliva was measured by calculation based on the mass change, and the mean value obtained was 3.47 μl (SD 0.49 μl, CV 14.2%). Finally, a calibration curve was produced for the whole system including the saliva transcription device and the analysis showed that $y = 13.58e^{0.0123x}$. At the same time, the salivary amylase activity slightly decreased to an R^2 value of 0.72 (Fig. 4) in the range from 57.6 to 423.5 kU/L.

3.2 *Stress evaluation*

Even in the prestress period, the minimum (43.2 kU/L) and maximum (286.6 kU/L) salivary amylase activities exhibited a difference of more than sixfold in the the case of six subjects. Although the subjects were maintained in a sitting position and relaxed while the saliva was collected, the salivary amylase activity varied between individuals. Correlations between salivary amylase activities among the prestress, midstress and poststress samples were then evaluated (Fig. 5(a)). The mean values were 147.2 for prestress, 203.8 for midstress and 153.4 kU/L for poststress (Table 1). For five of the subjects, the midstress salivary amylase activity was higher than the prestress value. The rate of increase was 34% on average, with a maximum of 108%. The Wilcoxon test was conducted on the prestress and midstress salivary amylase activities of five subjects, except for subject A. The results revealed that $p = 0.043 < 0.05$, and a significant difference between the two sets of samples was confirmed. Also, the result of the Wilcoxon test conducted on the values of the midstress and poststress salivary amylase activities showed $p = 0.043 < 0.05$ and again confirmed the significant difference between the two sets of samples.

The concentration of chemical substances contained in saliva can be influenced by the volume flow of saliva secretion. In order to cancel out this effect, we multiplied the saliva

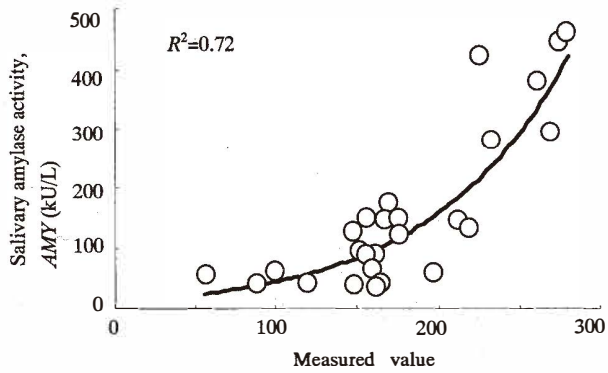

Fig. 4. Calibration curve of the fully constructed salivary amylase activity monitor.

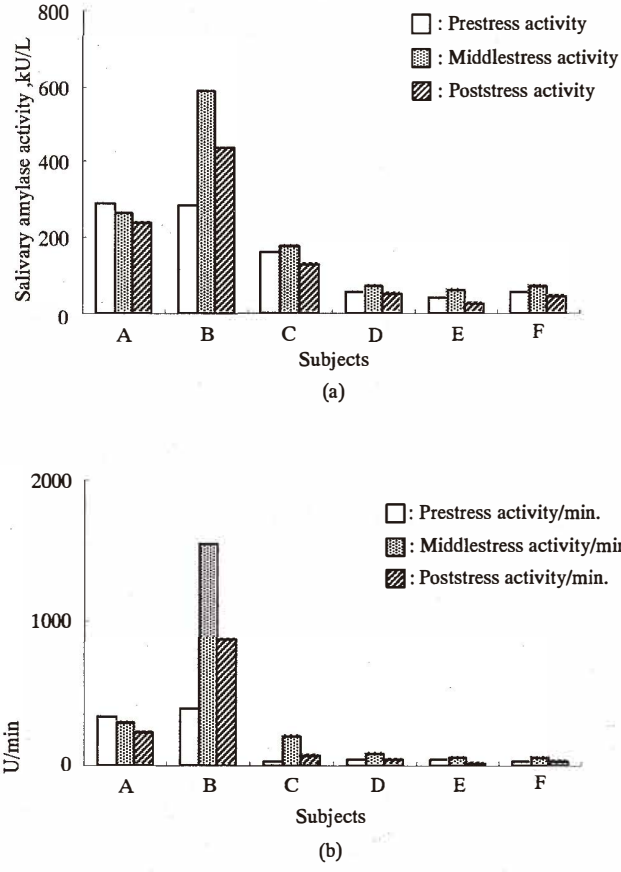

Fig. 5. Change in salivary amylase activity using a videotape of corneal transplant surgery as a mental stressor.

Table 1
Measurement results of the salivary amylase activity and its increasing rate.

Subjects	Salivary amylase activity *AMY*(kU/L)			Increasing rate (%, pre-middle)	Salivary amylase activityper min AMY_{min}(U/min)			Increasing rate (%, pre-middle)
	Pre	Mid	Post		Pre	Mid	Post	
A	286.6	260.0	238.1	–9	332.7	293.0	235.5	–12
B	282.7	588.0	433.7	108	393.7	1554.7	869.6	295
C	161.3	173.7	129.1	7	28.0	200.4	71.1	616
D	55.6	70.3	50.2	26	43.0	76.8	35.9	79
E	43.2	59.6	23.6	38	41.4	48.2	13.2	16
F	54.0	71.3	45.5	32	28.3	57.5	29.0	103
Mean± SD	147.2± 104.8	203.8± 186.1	153.4± 144.6	34±40	144.5± 155.7	371.8± 536.2	209.1± 304.6	183±238

secretion volume (ml/min) and salivary amylase activity activity (U/L) together so that the salivary amylase activity per minute (U/min) was calculated (Fig. 5(b)). The mean pre-, mid- and poststress salivary amylase activities per min were 144.5, 371.8 and 209.1 U/min, respectively (Table 1). The Wilcoxon test was also conducted on the prestress and midstress salivary amylase activity for five subjects, except for subject A. The result showed $p = 0.043 < 0.05$ and a positive difference was confirmed; the Wilcoxon test was also conducted on the midstress and prestress salivary amylase activities for the same subjects, resulting in $p = 0.043 < 0.05$, again showing the significant difference between them.

4. Discussion

The amylase activities for the calibration curve for the amylase activity testing tape ranged from 14.4 to 423.5 kU/L, with an R^2 value of 0.88 showing a favorable result. The salivary amylase activities that have been reported thus far range from some tens to some hundreds of kU/L.[11–14] Therefore, it was confirmed that the amylase activity testing tape covers a sufficiently wide measurement range. Based on these results, the feasibility of using the testing tape is confirmed. However, the following technical problems were revealed in the experiment:

1. When salivary amylase activity is higher than 200 kU/L, the calibration curve shows a saturated characteristic.
2. Since measurement errors dependent on reaction time were rather large, control of reaction time is crucial.

In order to improve the saturation characteristics, a competitive substrate against Ga1-G2-CNP could be added to suppress the side reaction. However, with increasing molecular weight, the substrate would experience additional enzymatic influence other than that of amylase. Therefore, to enhance α-amylase specificity, a suppressor substrate with a relatively small molecular weight, *e.g.*, an oligosaccharide such as maltopentaose, could be considered.

Moreover, we developed a saliva transcription device in order to control reaction time. With regard to the quantitative analysis of saliva, since 3.47 μl of saliva could be controlled with a CV of 14.2%, it was considered that the device could control relatively well saliva volume on the order of microliter. However, the calibration curve for the fully constructed salivary amylase activity monitor lowered to a state where the R^2 value was 0.72. In order to improve the performance of the system, it was considered that the transcription volume of saliva should be further reduced.

With regard to the stress evaluation, it was confirmed that increases in stress level could be tracked by an increase in salivary amylase activity, and a significant difference was confirmed between pre- and midstress salivary amylase activities. Also, because a significant difference exists between midstress and poststress salivary amylase activities, the easing of stress could be perceived by a decrease in salivary amylase activity.

The results obtained from the analysis of salivary amylase activity for 8 min before and after stress loading of 10 min suggested that changes in human stress could be analyzed within 10 min using our method. The response of salivary amylase activity to mental stress was interesting, and further study should be expected.

To cancel out the influence of the volume of saliva that was secreted, salivary volume and salivary amylase activity were multiplied and salivary amylase activity per minute was calculated. However, no significant difference was found in the relationship whether or not we considered the volume of saliva secretion at either pre- mid- or poststress. Although the influence of salivary secretion rate on salivary amylase activity could not be completely neglected, no significant influence was observed with respect to mental stress evaluation for the whole-saliva collection from healthy subjects under quiet conditions without stimulation.

5. Conclusion

To establish a method of evaluating human stress, we focused on salivary amylase activity and developed a disposable test strip and a saliva transcription device. A salivary amylase activity monitor consisting of a test strip, a saliva transcription device and an optical analyzer was fabricated. The experimental results revealed that the calibration curve of the salivary amylase testing tape ranged from 14.4 to 423.5 kU/L, with an R^2 value of 0.88. The testing tape could be used for the analysis of salivary amylase activity. Also, a calibration curve for the fully constructed salivary amylase activity monitor including the use of the amylase transcription device indicated an R^2 value of 0.72, which was a little low.

In order to evaluate the monitor, salivary amylase activity was measured using a videotape of corneal transplant surgery as a mental stressor. As a result, a significant difference in salivary amylase activity was recognized between the prestress, midstress and poststress periods, and it was confirmed that increasing or decreasing human stress levels could be perceived as increases and decreases in salivary amylase activity. Also it was suggested that changes in stress could be analyzed within a short period of 10 min. Although the influence of saliva secretion volume on salivary amylase activity could not be ignored, its influence should be minor for the evaluation of mental stress levels using this method.

Based on this study, we were able to develop a portable salivary amylase activity monitor that has the advantages of simplicity and which can immediately be used any time. In addition, we demonstrated that salivary amylase activity could be a useful index of human stress.

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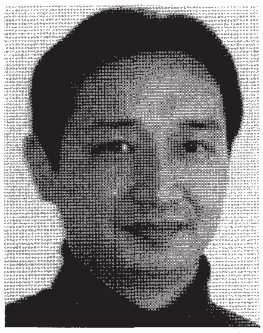
About the Authors



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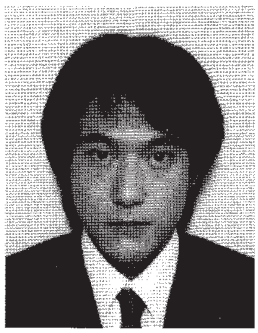

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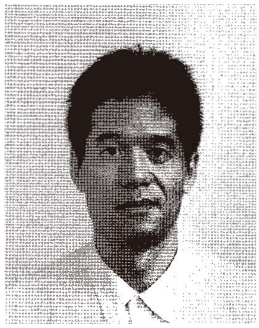

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