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Original Article

Evaluation of inhibitory activity, purification and X-ray crystallography of Alpha-Amylase inhibitor from *Phaseolus vulgaris* cultivars of Uttarakhand, India

Avaliação da atividade inibitória, purificação e cristalografia de raios X do inibidor Alfa-Amilase de cultivares de *Phaseolus vulgaris* de Uttarakhand, Índia

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Abstract

The present work is based on analysis of inhibitory activity of alpha-amylase inhibitor in selected cultivars of *Phaseolus vulgaris* of Uttarakhand. Fifteen samples were assessed for inhibitory activity of alpha-amylase inhibitor. Significant variations were found in different cultivars. Crude extract of alpha-amylase inhibitor from sample PUR (Purola) have shown maximum inhibitory activity (70.2 ± 0.84). Crude extract of all the cultivars have shown considerable variations in inhibitory activity in the temperature ranging from 20ºC to 100ºC. Based on inhibitory activity and heat stability profile, the alpha amylase inhibitor was purified from PUR cultivar. The purified inhibitor was found to be stable even at 90ºC with an inhibitory activity of 97.20 ±0.09. The molecular weight of purified inhibitor on Native PAGE (Polyacrylamide gel electrophoresis) was found to be 31kd, consisting of two subunits of 17kd and 14kd on SDS-PAGE.

Keywords: alpha-amylase inhibitor, *Phaseolus vulgaris*, inhibitory activity, PAGE, starch blocking activity, X-ray Crystallography.

Resumo

O presente trabalho é fundamentado na análise da atividade inibitória do inibidor da alfa-amilase em cultivares selecionadas de *Phaseolus vulgaris*, de Uttarakhand. Quinze amostras foram avaliadas quanto à atividade inibitória do inibidor da alfa-amilase. Variações significativas foram encontradas em diferentes cultivares. O extrato bruto do inibidor da alfa-amilase da amostra PUR (Purola) apresentou atividade inibitória máxima (70,2 ± 0,84). O extrato bruto de todas as cultivares apresentou variações consideráveis na atividade inibitória na temperatura de 20ºC a 100ºC. Com base na atividade inibitória e no perfil de estabilidade ao calor, o inibidor da alfa-amilase foi purificado do cultivar PUR. O inibidor purificado mostrou-se estável mesmo a 90ºC, com uma atividade inibitória de 97,20 ± 0,09. O peso molecular do inibidor purificado em Native PAGE (eletroforese em gel de poliacrilamida) foi de 31kd, consistindo em duas subunidades de 17kd e 14kd em SDS-PAGE.

Palavras-chave: inibidor de alfa-amilase, *Phaseolus vulgaris*, atividade inibitória, PAGE, atividade de bloqueio do amido, cristalografia de raio X.

1. Introduction

Plant seeds produce a variety of proteinaceous inhibitors of proteases and amylases. These inhibitors are characterized on the basis of sequences similarity and class of enzyme they inhibited (Clemente et al., 2019). Seed of common bean (*Phaseolus vulgaris* L.) contain certain inhibitors for digestive enzyme α-amylase. The amylase inhibitor does not inhibit the activity of plant, fungal and bacterial α- amylases, but inhibits the activity in mammals and some insects (Bahareh et al., 2016). The α-amylase inhibitor strongly inhibits the larval midgut α-amylase activities of adzuki bean weevil (*Collasobruchus chinesis* L.) and Cowpea weevil (*Collasobruchus maculatus*), non-pest species of common bean (Gupta et al., 2013). The amylase inhibitors can be classified according to their tertiary

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structure in six different classes, namely lectin like, knottin like, cereal type, kunitz like, γ-purothinin like and thaumatin like (Solanki et al., 2018). *Phaseolus vulgaris* α-amylase inhibitors are also known as starch blockers and has been developed into more effective control agents for diabetes and obesity (Li et al., 2020; Zhang et al., 2020).

A starch blocker is a substance that interferes with the breakdown of starch leading to reduced digestibility such that energy derived from the starch is reduced or rate of body absorption of energy in the form of glucose is reduced (Samtiya et al., 2020). Several amylase inhibitors drugs (acarbose, voglibose) are in use for diabetic patients, often in conjugation with insulin (Ghaedi et al., 2020). Although the biochemical properties of legume α-amylase inhibitor have been studied for over 20 years some discrepancies dealing with their physico-chemical and functional properties have been frequently reported. In addition, only a little is known on their structural features, and their inhibition mechanism remains to be studied in details. Amylase inhibitor has been shown to have nutraceutical properties as well (Yao et al., 2016). Since Uttarakhand is a rich repository of beans as more than 50 cultivars have been found, therefore the present study was undertaken to evaluate the starch blocking activity i.e. amylase inhibitor activity, heat stability of alpha-amylase inhibitor in selected cultivars, purification and crystallographic analysis of alpha-amylase inhibitor.

2. Materials and Methods

Seed samples: Seeds of *Phaseolus vulgaris* have been collected from different geographical locations of Uttarakhand. Fifteen cultivars showing variation in seed coat colour, size and shape were selected. The seeds were authenticated and deposited in National Bureau of Plant and Genetic Research, New Delhi, India (Table 1).

Table 1. List of *Phaseolus vulgaris* cultivars collected from different provenances of Uttarakhand.

Code	Location	Accession No.
PUR	Purola	IC-569208
DHA	Dhankot	IC-569215
TAP	Tapovan	IC-569214
MUN-1	Munsiyari	IC-569213
DUN	Dunagiri	IC-582575
DWA	Dwarahaat	IC-582576
HAR	Harsil	IC-569211
JOSH	Joshimath	IC-582574
CHM	Chamba	IC-569209
MAJ	Majkhali	IC-569212
CHK	Chakrata	IC-582573
MUN-2	Munsiyari	IC-569210
RUD	Rudraprayag	IC-582577
ALM	Almora	IC-582578
RAM	Ramgarh	IC-582572

Chemicals and Reagents: Sephadex G-50, PPA (Porcine pancreatic amylase) were purchased from Sigma (India). Protein markers were purchased from Genei (India) and others required chemicals from Himedia (India).

Crude extract Preparation: The extraction of seed proteins from seed flour was done according to the method described by literature[3]. 100 mg of finely grounded seed flour was taken, homogenised in extraction buffer and was incubated at 4ºC for 1hr. The homogenate was then centrifuged at 15000 rpm for 15 min at 4ºC. The supernatant was collected and stored in aliquots at -20°C for further analysis. The protein content was measured by method described by Bradford (Mumbarkar and Shravya, 2013).

Amylase inhibitory activity: The amylase inhibitory activity was determined according to literature descriptions (Yao et al., 2016) with some modifications. A soluble starch solution (0.4 ml,1% w/v) was made in 80mM phosphate buffer (pH = 6.9) and a solution of PPA (0.2 ml, 0.001% w/v) in 20 mM acetate buffer (pH-4.5, containing 20mM $CaCl_2$ and 10 mM NaCl) was added into it and then incubated for 15 min at 37ºC. The reaction was stopped by addition of 0.8ml of Dinitrosalicylic acid reagent (1gm DNS, 200 mg crystalline phenol and 50 mg of sodium sulphite dissolved in 1% NaOH). The contents were heated in a boiling water bath for 5 min, and after cooling it was diluted with 4ml of water. Absorbance of the mixture was read at 540 nm against blank prepared without using PPA. Amount of maltose produced was calculated from standard curve of maltose. The above method was also used to describe α-amylase inhibitor activity but PPA solution and purified inhibitor solutions (0.2ml) were pre-incubated for 15 min before addition of soluble starch solution. Alpha-amylase inhibitory activity was calculated according to Equation 1 shown below:

$$Inhibitory\ activity\ (\%) = \left[M_o - M_i / M_o\right] x\ 100 \quad (1)$$

Where, M_o and M_i are amount of maltose (mg/ml) produced in absence and presence of inhibitor respectively, under the same conditions.

Heat stability: Heat stability was evaluated from literature descriptions (Fernando et al., 2019). Both the extracts (crude and purified) were incubated in a water bath at different temperatures ranges from 20ºC to 100ºC with the difference of 10ºC, after that amylase inhibitory activity was calculated

Purification of α-amylase inhibitor: Ammonium sulphate precipitation (80-100% saturation) of the crude protein extract was performed at 4°C. The precipitate was dissolved in 10mM Tris-HCl and was dialyzed against buffer in batches. The dialyzed material was stored at -20ºC till further analysis. The α-amylase inhibitor was fractionated by repeated size exclusion chromatography on a Sephadex G-50 column (26 x 1.2 cm).

Molecular identification: The polypeptides in the samples were fractionated using SDS-PAGE (15%) under reducing conditions and Native PAGE. The molecular weight of purified inhibitor was determined by using medium range protein marker (14.3 - 97.4 kd).

X-ray Crystallography: The purified protein sample was re-dissolved at a concentration of 10 mg/ml in double-deionised water. Crystallization was performed using VDX48 plates by the hanging-drop vapour-diffusion method.

Statistical analysis: Each sample was analysed in triplicates and the values were averaged. Data was assessed by analysis of variance (ANOVA), previous verification of normal distribution and variance homogeneity (Zar, 1999; Ostergova and Ostertag, 2013) and mean comparison was done by using Duncan's multiple range test using software R (R Development Core Team, 2009).

3. Results

Assessment of starch blocking activity of α-amylase inhibitor: Alpha-amylase inhibitor protein inhibits the α-amylase enzyme and interferes in digestion of starch. Therefore, inhibitory activity of α-amylase inhibitor in selected cultivars can be used as a measure of starch blocking activity. Inhibition of pancreatic amylase was observed in all the seed samples thus showing the presence of α- amylase inhibitor (Table 2). The cultivars were found to differ significantly in inhibitory activity. The maximum inhibitory activity was found to occur in sample PUR (70.2 ± 0.84%) and minimum in sample DUN (39.43 ± 0.47%). Sample PUR and MUN-2 have not shown a significant difference in inhibitory activity, similarly sample MAJ and RUD have nearly similar inhibitory activity, whereas all other cultivars are found to differ significantly in inhibitory activity of amylase inhibitor.

Table 2. Inhibitory activity (% inhibition) of alpha-amylase inhibitor in Cultivars of *Phaseolus vulgaris*.

S.no	Cultivars	Inhibitory Activity (%)
1	DWA	$45.23^{a} \pm 0.23$
2	HAR	$52.69^{b} \pm 0.56$
3	CHM	$57.01^{g} \pm 0.78$
4	DUN	$39.43^{e} \pm 0.47$
5	PUR	$70.2^{c} \pm 0.84$
6	DHA	$68.56^{d} \pm 0.65$
7	JOSH	$65.23^{f} \pm 0.05$
8	MUN-1	$55.92^{k} \pm 0.15$
9	TAP	$56.17^{h} \pm 0.10$
10	MAJ	$60.21^{k} \pm 0.08$
11	CHK	$58.45^{g} \pm 0.36$
12	ALM	$64.56^{f} \pm 0.18$
13	RUD	$61.58^{k} \pm 0.53$
14	RAM	$66.34^{f} \pm 0.46$
15	MUN-2	$69.57^{dc} \pm 0.18$

Means in a column with different letters are significantly different ($p<0.05$: n=3).

Effect of temperature on α-amylase inhibitor: Significant variations in inhibitory activity were found between all the cultivars. The inhibitory activity of cultivars increases upto 60 or 70ºC, afterwards there is decrease in the values of inhibitory activity. Sample DWA, MAJ, ALM, RAM and MUN-2 has shown maximum inhibitory activity at 50ºC and above this temperature the inhibitory activity decreases. Similarly, sample CHM, DUN, DHA, JOSH, TAP, MUN-1, MAJ and CHK have shown an increase in activity upto 60ºC and then there is decrease in activity with an increase in temperature (Table 3). The minimum inhibitory activity at 20ºC was shown by sample DUN (36.45 ± 0.65%) and maximum by sample PUR (71.34 ± 0.42%) than other cultivars. On the other hand, at 100ºC the minimum inhibitory activity was shown by sample DWA (25.22 ± 0.40%) and maximum by sample PUR (70.23 ± 0.28%). Out of the fifteen cultivars, sample PUR has shown a consistent stability in amylase inhibitor activity even at 100ºC (Table 4).

X-ray crystallography of purified α-amylase inhibitor: Crystals of purified enzymes from PUR cultivars were obtained by hanging drop methods and was found to differ in shape, wavelength, and space groups and in solvent content. X-ray analysis of sample- PUR was purified to homogeneity and crystallized at 293 K (Figure 1). The crystals diffracted beyond 1.0 Å resolution using synchrotron single beam x-ray crystallography. The crystal belongs to the monoclinic space group $P2_12_12$, with Unit-cell parameters (A°) a = 74.56, b = 60.45, c = 64.40. Percent solvent content was 42.05%.

Purification and Molecular weight determination of α-amylase inhibitor: Based upon the inhibitor activity and effect of temperature on crude extract of amylase inhibitor in selected cultivars, purification of amylase

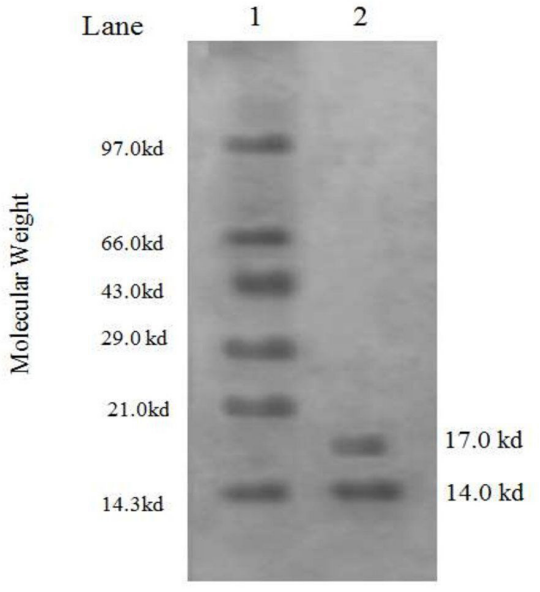

Figure 1. X-ray crystallographic analysis of purified inhibitor from Cultivar-PUR.

Table 3. Effect of temperature on inhibitory activity of alpha-amylase inhibitor (different letters are significantly different ($p<0.05$: n=3).

Cultivars	Inhibitor activity (%)								
	20	30	40	50	60	70	80	90	100
DWA	46.23^{a}±0.80	50.34^{b}±0.17	52.33^{cb}±0.28	56.43^{d}±0.56	55.87^{e}±0.70	50.22^{b}±0.20	40.34^{f}±0.32	36.34^{g}±0.15	25.22^{h}±0.40
HAR	47.23^{a}±0.19	53.34^{b}±0.61	56.75^{c}±0.79	60.45^{d}±0.69	64.23^{e}±0.25	68.34^{f}±0.48	62.14^{g}±0.77	56.77^{c}±0.11	50.13^{h}±0.16
CHM	52.43^{a}±0.46	56.71^{b}±0.22	60.34^{c}±0.18	62.56^{d}±0.08	68.45^{e}±0.81	64.56^{f}±0.38	60.35^{c}±0.93	57.25^{g}±0.79	50.24^{h}±0.10
DUN	36.45^{a}±0.65	40.56^{b}±0.47	45.67^{c}±0.37	49.11^{dc}±0.06	53.78^{d}±0.14	50.24^{d}±0.24	47.88^{c}±0.13	42.19^{b}±0.12	34.55^{a}±0.51
PUR	71.34^{a}±0.42	67.82^{b}±0.40	68.24^{d}±0.50	71.34^{ca}±0.36	71.64^{c}±0.29	74.45^{e}±0.53	75.97^{f}±0.66	75.23^{e}±0.22	70.23^{h}±0.28
DHA	67.64^{a}±0.07	67.45^{ab}±0.39	69.67^{b}±0.67	70.23^{d}±0.38	71.64^{e}±0.67	63.45^{f}±0.25	61.63^{g}±0.31	55.56^{h}±0.86	50.23^{i}±0.92
JOSH	67.04^{a}±0.65	69.76^{b}±0.56	71.45^{c}±0.49	72.23^{c}±0.38	71.23^{b}±0.96	69.16^{c}±0.24	68.53^{b}±0.69	66.23^{ak}±0.2	65.80^{k}±0.12
TAP	57.62^{a}±0.02	57.40^{ac}±0.74	59.63^{d}±0.55	62.43^{f}±0.41	61.23^{f}±0.18	58.45^{adc}±0.20	54.12^{g}±0.47	50.62^{h}±0.18	45.33^{i}±0.60
MUN-1	56.52^{a}±0.61	56.87^{b}±0.06	58.62^{c}±0.60	60.45^{c}±0.52	61.72^{d}±0.24	58.23^{b}±0.27	52.45^{e}±0.81	49.25^{f}±0.93	46.54^{g}±0.01
MAJ	60.41^{a}±0.18	60.55^{a}±0.10	67.22^{b}±0.48	69.13^{c}±0.20	65.18^{d}±0.05	62.38^{e}±0.18	51.87^{f}±0.11	42.14^{g}±0.14	38.12^{h}±0.45
CHK	57.12^{a}±0.14	60.14^{b}±0.20	62.23^{c}±0.08	65.18^{d}±0.10	67.25^{e}±0.45	63.30^{c}±0.23	60.12^{b}±0.43	58.55^{a}±0.80	52.16^{f}±0.26
ALM	65.02^{a}±0.67	67.34^{b}±0.14	69.24^{c}±0.45	72.67^{d}±0.78	70.92^{e}±0.34	68.14^{b}±0.56	64.36^{a}±0.77	62.08^{f}±0.29	56.68^{g}±0.05
RUD	60.34^{a}±0.46	61.86^{b}±0.24	67.98^{c}±0.04	70.24^{d}±0.85	70.17^{d}±0.48	68.34^{e}±0.37	64.22^{f}±0.28	60.56^{a}±0.40	56.74^{g}±0.17

Table 4. Effect of temperature on purified α-amylase inhibitor from cultivar PUR.

Temperature (ºC)	Inhibitory activity (%)
20	94.30^{a} ± 0.55
30	94.88^{a} ± 0.34
40	95.67^{ca} ± 0.20
50	96.34^{d} ± 0.15
60	97.56^{e} ± 0.85
70	98.56^{g} ± 0.72
80	98.23^{g} ± 0.03
90	97.20^{eg} ± 0.09
100	96.87^{he} ± 0.47

Means in a column with different letters are significantly different ($p<0.05$: n=3).

inhibitor was done from sample PUR. Purification was done by ammonium salt precipitation (80-90%) followed by dialysis and gel-filtration chromatography using Sephadex G-50 column. The fractions were collected at constant flow rate and were assayed for protein estimation (%) and specific activity (%). The specific activity was found to increase after each purification procedure (Table 5). The fractions eluted from sephadex column were analysed for inhibitory activity against PPA (Figure 2) The purified fraction of sample PUR on SDS-PAGE was found to resolve into two bands of molecular weight of 14 and 17kd (Figure 3). These bands may be due to denaturation of pure amylase inhibitor into two subunits. Native PAGE of purified inhibitor from sample-PUR has shown a single band corresponding to molecular weight of 31kd (Figure 4).

Table 5. Purification of alpha-amylase inhibitor from cultivar-PUR.

Sample	Inhibitory activity (%)	Protein content (%)	Specific activity (%)	Fold Purification
Crude extract	70.67	18.2	3.88	1.0
Amm. ppt. (80-90%)	80.45	10.56	7.60	2.0
Sephadex G-50	94.25	7.36	12.8	3.3

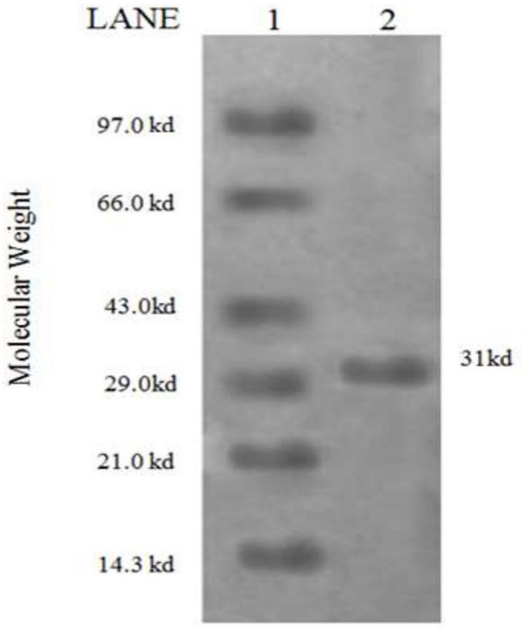

Figure 2. Medium Range Protein Marker (14.3-97.0 kd).

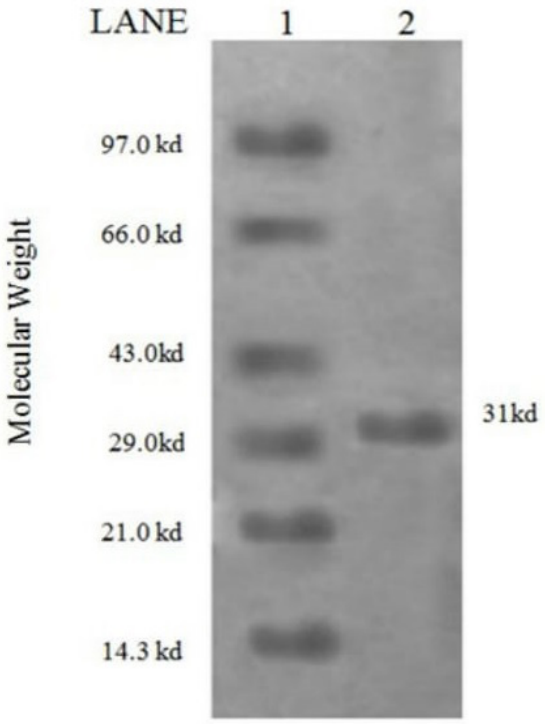

Figure 3. Purified α-amylase inhibitor from cultivar PUR.

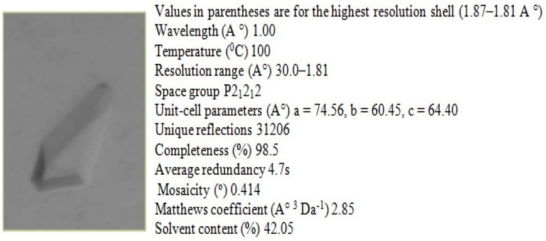

Figure 4. Native Page Analysis of purified α- amylase inhibitor from Cultivar PUR.

4. Discussion

The literature about of assessment of starch blocking activity of α-amylase inhibitor into the action mechanism of the *Phaseolus vulgaris* shows that the inhibitor is effective in preventing starch digestion by restricting access to active site of the enzyme. The molecular-level binding of the action of the amylase inhibitor on human pancreatic amylase and PPA was reviewed in detail (Da Lage, 2018). The study reveals that during inhibition, the components of inhibitor molecule play an important role in this mechanism. The main components that participate in the mechanism include two loops of the inhibitor made up of residues 20 - 45 and 172 – 190 (Ibrahim et al., 2017), the amylase domains A/B and the active site non-loop residues (Asp197, Glu233, Asp300 and Arg74) in human pancreatic amylase (Koukiekolo et al., 2001)

Heat stability of α-amylase inhibitor has been shown by many studies, the literature about inhibitor has been found to be stable at a temperature range of 40-90ºC (Ninomiya et al., 2018; Fernando et al., 2019). The inhibitor is completely inactivated at 100ºC by boiling for 10 min (Yao et al., 2016). The results of purification and molecular weight determination of α-amylase inhibitor obtained in present study were similar with literature references (Yao et al., 2016) where the molecular weight of α-amylase inhibitor from *Vigna sublobata* was found to be 14kd on SDS-PAGE.

Similar type of crystallography of α-amylase inhibitor has been reported by literature references (Lin et al., 2006) in bifunctional amylase/subtilisin inhibitor purified from rice in which the crystal was found to be monoclinic with unit cell parameters of a = 79.99, b = 62.95, c = 66.70 Å.

As conclusion, the present work describes the comparative analysis of alpha amylase inhibitor activity in selected cultivars of kidney beans and its purification from sample PUR. The present study revealed that the inhibitory activity of plant alpha- amylase inhibitor against mammalian amylases could cause a marked decrease in the availability of digested starch. This could suggest a potential in the prevention and treatment of diabetes and nutritional problems, which result in obesity. Based on the results of this study, the α-amylase inhibitors from *Phaseolus vulgaris* may have potential in the prevention and therapy of obesity and diabetes.

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