Antioxidant and antimicrobial activities of Penicillium sp. lectins

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Abstract: Lectins are a diverse group of proteins of non-immune origin that interact specifically with glycans. Owing to their specificity, they can mediate various cellular and molecular recognition processes. To explore information on biological activities of lectins from *Penicillium duclauxii*, *P. proteolyticum* and *P. griseoroseum*, they were investigated for their antioxidant and antimicrobial activities. *Penicillium* sp. lectins exhibited moderate antioxidant activity. *P. duclauxii*, *P. proteolyticum* and *P. griseoroseum* lectins inhibited DPPH with an IC₅₀ value of 71.42, 75.04 and 82.11 µg/mL, respectively. *P. duclauxii*, *P. proteolyticum* and *P. griseoroseum* lectins inhibited the hydrogen peroxide radical with IC₅₀ values of 198.57, 209.76 and 215.31 µg/mL, respectively. *P. duclauxii* and *P. proteolyticum* lectins exhibited potent antibacterial activity against Gram-negative bacteria. *P. griseoroseum* lectin inhibited only Gram-positive bacteria. *Penicillium* sp. lectins will help to understand their biomedical applications. This is the first report on the antioxidant and antimicrobial activities of purified lectins from *Penicillium* sp.

Keywords: Penicillium; lectin; antioxidant; antibacterial; antifungal

INTRODUCTION

Lectins are multivalent glycoproteins that possess two or more carbohydrate binding sites. They are of nonimmune origin [1] and bind reversibly and specifically to free or membrane bound carbohydrate moieties [1]. Lectins are widely distributed in nature and found in all life forms including plants [2], animals [3], algae [4,5], cyanobacteria [6,7], yeasts [8], mushrooms [9] and microfungi [10]. Amongst the microfungi, lectins from Penicillium sp. [11,12], Fusarium sp. [13] and Aspergillus sp. [14,15] have been widely reported. Lectins from diverse sources are involved in cell-cell/hostpathogen interactions and trigger many of reactions [16-18]. They possess the ability to induce agglutination of cells and have been explored for their biomedical potential [19]. They manifest diverse biological activities such as mitogenic [20-22], antiproliferative [23,24], immunomodulatory [25,26], antitumor [27], antimicrobial [28,29], antioxidant [30,31], etc.

Antioxidants are molecules that donate electrons/ hydrogen to radicals and quench free radical reactions based on their reduction potential or other traits. During normal or pathological cell metabolism, free radicals are generated that have one or more unpaired electrons (superoxide, hydroxyl, peroxyl), and the compounds that have the potential to scavenge free radicals can play a role in curing diseased cells and improving pathological conditions. Thus, they play a significant role in defense mechanisms of organisms against various pathologies related to free radical attack [32]. There has been an increase in research into new compounds with antioxidant activity in natural products. Many plant lectins have been reported to possess an antioxidant potential [23,27,28]. However, there is only one report on the antioxidant potential of lectins from microfungi [33].

Lectins from various sources have also been explored for their potential antimicrobial activity [34]. The carbohydrate-binding sites of lectins can interact with carbohydrates present on the surface of microorganisms and exert a antimicrobial effect. Thus, different carbohydrate specificities of lectins greatly influence recognized targets [35]. There are only a few reports on the antimicrobial action of lectins from microfungi. The antimicrobial activities of lectins from *Fusarium* sp. [36], *Aspergillus* sp. [37,38] and *Penicillium* sp. [39] have been reported. New lectins have been reported from *Penicillium* sp. [11,12,39], however, scant information is available in the literature regarding their biological potential. In previous studies, lectins from *Penicillium* sp. were purified, characterized and evaluated for their mitogenicity [40,41]. The aim of the current study was to explore the antioxidative and antimicrobial activities of these purified *Penicillium* sp. lectins.

MATERIALS AND METHODS

Ethical statement

Animal care was taken according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of the Government of India. Experimental work was approved by the Institutional Ethics Committee for Animals (Permit No. 107/99/CPCSEA/201429).

Procurement and maintenance of test organisms used for antimicrobial activity

Cultures used for the antibacterial assay were Grampositive bacteria (Bacillus cereus MTCC 1306 and Staphylococcus aureus MTCC 6908) and Gram-negative bacteria (Pseudomonas aeruginosa MTCC 1034 and Escherichia coli MTCC 64). Test organisms used for the antifungal assay were Saccharomyces cerevisiae MTCC 170, Candida albicans MTCC 183, Aspergillus flavus MTCC 277 and Fusarium oxysporum MTCC 284. All cultures were procured from MTCC, IMTECH Chandigarh, India, and maintained on prescribed media. Bacterial cultures were maintained on nutrient agar slants containing (expressed as percentage (%) of contents): 0.1 beef extract, 0.2 yeast extract, 0.5 peptone, 0.5 NaCl and 3.0 agar. Yeast cultures (S. cerevisiae and C. albicans) were maintained on yeast extract peptone dextrose (YEPD) agar slopes containing (in %): 0.3 yeast extract, 1.0 peptone, 2.0 dextrose and 3.0 agar. A. flavus was maintained on Czapek yeast agar (CYA) slants containing (in %): 0.1

 K_2 HPO₄, 0.5 yeast extract, 3.0 sucrose, 0.3 NaNO₃, 0.05 KCl, 0.05 MgSO₄7H₂O, 0.001 FeSO₄7H₂O and 3.0 agar. *F. oxysporum* was maintained on potato dextrose agar (PDA) slants containing (in %): potato extract 20.0, dextrose 2.0, agar 3.0 and pH adjusted to 5.6.

Extraction and purification of *Penicillium* sp. lectins

Fungal cultures of *P. duclauxii* MTCC 7997, *P. proteolyticum* MTCC 10300 and *P. griseoroseum* MTCC 9624 were obtained from MTCC housed at IMTECH, Chandigarh, India. All the cultures were maintained and cultivated on their respective medium under growth conditions specified by MTCC, as described earlier [12]. Fungal mycelia were harvested by filtration from broth cultures and intracellular *Penicillium* sp. lectins were extracted in 0.1 M phosphate buffered saline (PBS), pH 7.2. as described [14]. *Penicillium* sp. lectins were purified by ion chromatography on a DEAE Sepharose column (GE Healthcare, USA) and gel exclusion chromatography on a Sephadex G-100 column following the reported methodology [40,41].

Hemagglutination assay and protein quantification

Rabbit erythrocytes were used for the hemagglutination assay with *Penicillium* lectins as described [14]. Hemagglutination titer was used to express lectin activity, which is defined as the reciprocal of the highest dilution that exhibits visible erythrocyte hemagglutination. Protein quantification of *Penicillium* sp. lectins was performed by the Lowry method [42]. Purified lectins were then evaluated for their antioxidant potential and antimicrobial activity.

DPPH (2,2-diphenyl-1picrylhydrazyl) radical scavenging assay

The antioxidant activity of *Penicillium* sp. lectins was determined by DPPH radical scavenging [43]. The radical scavenging effect of lectins was studied using DPPH, a stable free radical. The DPPH-based photometric measurement is widely used for antioxidant screening as it is a simple and rapid method. A purified *Penicillium* lectin solution (0.1 mL) in phosphate buffer at different concentrations (1, 5, 10, 25, 50, 75, 100 μ g/mL) was added to a methanolic solution of

DPPH (3.9 mL). The reaction mixture (4 mL) was shaken vigorously and then incubated for 30 min in the dark at room temperature. Absorbance of the resultant solution was read spectrophotometrically at 517 nm against the blank (methanol plus lectin solution). The control solution contained all reagents except the test sample. Owing to the strong reducing power and weak metal chelating ability, ascorbic acid at different concentrations (1, 5, 10, 25, 50, 75, 100 μ g/ mL) served as a standard. The DPPH radical scavenging activity of lectins was determined by converting the absorbance into the percentage antioxidant activity using the following equation:

Percent inhibition (%) = $\frac{Abs_A - Abs_B}{Abs_A} \ge 100$

where Abs_A is the absorbance of the control reaction and Abs_B is the absorbance of the lectin solution.

The concentration of the test sample required to inhibit 50% of the DPPH free radical is defined by the IC_{50} (half maximal inhibitory concentration) value. Higher free radical (antioxidant) activity is indicated by lower IC_{50} values of the reaction mixture.

Hydrogen peroxide radical scavenging assay

The ability of *Penicillium* sp. lectin solution to scavenge hydrogen peroxide radicals was determined as described [44]. A 43-mM H_2O_2 solution was prepared in phosphate buffer (0.1 M, pH 7.4). Purified *Penicillium* sp. lectin solutions (3.4 mL) in phosphate buffer at different concentrations (50, 70, 100, 125, 150, 175, 200, 225, 250 µg/mL) were added to the H_2O_2 solution (0.6 mL, 43 mM). After 10 min, the absorbance of the H_2O_2 was read spectrophotometrically at 230 nm against the blank (phosphate buffer without H_2O_2). Ascorbic acid at different concentrations (50, 70, 100, 125, 150, 175, 200, 225, 250 µg/mL) was used as the standard for comparison. The hydrogen peroxide radical scavenging activity of lectins was then determined using the following equation:

Percent of H₂O₂ radical scavenging

activity (%) =
$$\frac{Abs_A - Abs_B}{Abs_A} \times 100$$

where Abs_A is the absorbance of the control reaction and Abs_B is the absorbance of the lectin solution. The half maximal inhibitory concentration (IC_{50}) was determined as described above.

Antimicrobial assay

The antimicrobial activity of purified *Penicillium* sp. lectins was assessed against various bacterial and fungal cultures by the disk diffusion method [45].

Antibacterial assay

The bacterial cell suspension was prepared by inoculating the test culture strains from nutrient agar slants into nutrient broth medium and grown for 24 h by shaking (150 rpm) at 37°C. The overnight-grown bacterial cultures were adjusted with sterile PBS (pH 7.4) to yield a 0.5 McFarland suspension. The prepared bacterial inoculum (100 µL) was then spread on Muller-Hinton agar plates by the three-dimension swab technique. Sterile filter paper disks (6.0 mm diameter, Whatman, U.K.) were placed on the inoculated agar plates and each disk was impregnated with purified Penicillium sp. lectin (50 µg). Inoculated agar plates were then incubated at 37°C for 24 h. Reference antibiotics, ciprofloxacin (10 μ g) and gentamicin (50 μ g), served as a positive control. The degree of susceptibility of the test organism was determined by examining the zone of inhibition around the sample disk. Bacterial growth encircled around the disks indicated the non-inhibitory action of lectin, whereas crescents of inhibition around disks depicted antibacterial activity.

Antifungal assay

Yeast cultures were grown in YEPD broth for 48 h at 30°C. Fungal cultures of *A. flavus* and *F. oxysporum* were grown on CYA and PDA, respectively, for 5 days at 25°C. The fungal suspensions (10^7 spores/mL) and yeast cells (10^7 cells/mL) were prepared, and spreading was performed on 2% (w/v) malt extract agar (MEA) plates. Purified *Penicillium* sp. lectins (50 µg) were applied on sterile filter paper disks as described above. Nystatin (100 units) and amphotericin B (50 µg) were used as the positive control. The zone of inhibition around the sample disk was detected after incubation for 72 h at 30°C for determination of the inhibitory potential.

Minimum inhibitory concentration (MIC) determination for antibacterial activity

Purified *Penicillium* sp. lectins were serially diluted with sterile water to different concentrations (5-50 μ g) for the determination of MIC. Bacterial cultures (100 μ L) were spread over Muller-Hinton agar plates. Different lectin concentrations were impregnated over sterile filter paper disks placed on agar plates. The disk diffusion method was performed to determine the inhibition of microorganisms. The MIC was taken to be the lowest concentration that inhibited bacterial growth.

Statistical analysis

All the experiments were repeated three times and the results are expressed as the mean±standard deviation of triplicate values.

RESULTS

Lectins from *P. duclauxii*, *P. proteolyticum* and *P. griseoroseum* were purified to the following specific activities: 131.1, 168.42 and 124.27 titer/mg, respectively, as described previously [40,41]. All lectins were used for determination of the antioxidant potential, and antifungal and antibacterial activities.

DPPH free radical scavenging by *Penicillium* sp. lectins

To evaluate the antioxidant activity, the DPPH free radical scavenging assay is considered the most accurate screening method. The percentage inhibition of DPPH by purified *Penicillium* sp. lectins and ascorbic acid (standard) was determined (Table 1). *Penicillium* sp. lectins possess a moderate antioxidant capacity. The highest scavenging action was shown by *P. duclauxii* lectin. IC_{50} values of 71.42 µg/mL, 75.04 µg/mL and 82.14 µg/mL were shown by *P. duclauxii*, *P. proteolyticum* and *P. griseoroseum* lectins, respectively, whereas ascorbic acid had an IC_{50} value of 48.80 µg/mL.

Hydrogen peroxide scavenging by *Penicillium* sp. lectins

The percentage inhibition of hydrogen peroxide by purified *Penicillium* sp. lectins and the standard,

ascorbic acid, were also determined (Table 2). The IC₅₀ values of *P. duclauxii*, *P. proteolyticum* and *P. griseoroseum* lectins for hydrogen peroxide were found at concentrations of 198.57 µg/mL, 209.76 µg/mL and 215.31 µg/mL, respectively. Ascorbic acid was used as the standard as it is a very strong antioxidant, and 50% scavenging of hydrogen peroxide radicals was achieved at a concentration of 150 µg/mL (IC₅₀).

Antimicrobial activity of Penicillium sp. lectins

The purified Penicillium sp. lectins were screened for antimicrobial activities by the disk diffusion method. The zone of inhibition by *Penicillium* sp. lectins was determined by measuring the diameters of the inhibitory zones (Table 3). P. duclauxii lectin exhibited an enhanced inhibitory effect over Gram-negative bacteria as compared to Gram-positive bacteria. A high antibacterial activity of P. duclauxii lectin was observed against E. coli and P. aeruginosa, with maximum zones of inhibition of 38±0.07 mm and 35±0.13 mm, respectively. P. proteolyticum lectin showed inhibitory zones of 27±0.88 mm and 22±0.19 mm against E. coli and P. aeruginosa, respectively. P. duclauxii lectin expressed low antibacterial activity against S. aureus (15±0.24 mm), whereas P. proteolyticum lectin was non-inhibitory against it. Growth of B. cereus remained unaffected with both lectins. However, P. griseoroseum lectin strongly inhibited Gram-positive bacteria, including S. aureus (27±0.39) and B. cereus (28±.43), but was non-inhibitory towards Gram-negative bacteria.

The growth of yeast (*C. albicans, S. cerevisiae*) and fungi (*A. niger, F. oxysporum*) was not affected in the assay. The MIC of purified *Penicillium* sp. lectins against bacterial strains was determined (Table 4). In the case of *P. duclauxii* lectin, the MIC was lowest for *E. coli* (7.5 μ g) and highest for *S. aureus* (45 μ g), whereas, *P griseoroseum* lectin had an MIC of 17.5 μ g towards *B. cereus*.

DISCUSSION

Oxidation processes associated with aerobic life forms can lead to cell and tissue damage as the result of the generation of increased amounts of reactive oxygen species (ROS) [46]. Antioxidants delay or inhibit this oxidation process and thus play a significant role in

Concentration (µg/mL)	Scavenging activity (%)				
	Ascorbic acid	P. duclauxii lectin	P. proteolyticum lectin	P. griseoroseum lectin	
1	15.22 ± 0.02	35.71±0.01	19.05 ± 0.01	20.05 ± 0.04	
5	25.36±0.04	37.77±0.04	23.63±0.02	22.36±0.03	
10	32.6±0.03	40.06±0.01	29.06±0.01	25.12±0.02	
25	41.13±0.04	42.84±0.02	37.48 ± 0.04	31.21±0.04	
50	58.82 ± 0.03	46.8±0.01	41.48 ± 0.03	40.88±0.03	
75	65.17±0.02	52.01±0.03	50.02 ± 0.02	47.02 ± 0.02	
100	71.32±0.01	54.28 ± 0.03	57.27±0.01	55.33±0.01	
IC ₅₀	48.80	71.42	75.04	82.14	

Table 1. DPPH radical scavenging by the standard (ascorbic acid) and purified Penicillium sp. lectins.

Table 2. Hydrogen peroxide radical scavenging by the standard (ascorbic acid) and purified Penicillium sp. lectins.

Concentration	Concentration Scavenging activity (%)			
(µg/mL)	Ascorbic acid	P. duclauxii lectin	P. proteolyticum lectin	P. griseoroseum lectin
50	32.22±0.01	9.2±0.04	$8.16 {\pm} 0.04$	10.16 ± 0.04
70	35.51±0.03	11.11 ± 0.01	19.05 ± 0.02	17.05 ± 0.03
100	38.53±0.02	14.28 ± 0.04	28.89±0.01	22.99±0.02
125	44.62 ± 0.04	26.34±0.02	31.41±0.03	27.21±0.04
150	51.49±0.03	39.04±0.03	34.33±0.02	32.43±0.02
175	55.31±0.01	43.8±0.04	41.21±0.01	40.11 ± 0.04
200	60.79 ± 0.04	51.74±0.02	45.71±0.04	48.66±0.01
225	63.47±0.02	58.09±0.03	54.55 ± 0.02	51.37±0.02
250	67.77±0.04	64.76±0.04	60.03 ± 0.04	59.22±0.04
IC ₅₀	150.162	198.57	209.76	215.3

the organism's defense against pathologies related to high ROS levels [32]. DPPH is a widely used assay for determining antioxidant activity [47]. *P. duclauxii, P. proteolyticum* and *P. griseoroseum* lectins exhibited concentration-dependent antioxidant activities. Interaction between lectin molecule (antioxidant) and DPPH radical can lead to a decrease in absorbance of DPPH radicals owing to scavenging of the radical by hydrogen donation [33]. *Penicillium* sp. extract was reported to express a DPPH free radical scavenging activity of about 50% at a concentration of 300 µg/mL [48]. Concentration-dependent free radical scavenging of DPPH is expressed by some endophytic fungal lectins [33].

P. duclauxii, P. proteolyticum and *P. griseoroseum* lectins exhibit antioxidant activities. The highest hydrogen peroxide scavenging activity was shown by *P. duclauxii* lectin. Hydrogen peroxide is a non-radical derivative of oxygen and can inactivate some enzymes. It is a weak oxidizing agent and can oxidize essential thiol (-SH) groups. It can react with different molecules in living organism after entry into cells

[49]. A concentration-dependent hydrogen peroxide scavenging activity was also displayed by lectins from endophytic fungi [33]. *Cratylia mollis* (pCramoll) and recombinant Cramoll 1 (rCramoll 1) lectins exhibited concentration-dependent attenuation of H_2O_2 induced oxidative stress [49]. Thus, antioxidants have gained interest owing to their role in preventing oxidative-stress related diseases caused by free radical attack on key biological components, such as nucleic acids and lipids [30].

Microorganisms usually possess carbohydrates on their cell surface that are either covalently bound (glycosylated teichoic acids to peptidoglycan) or noncovalently bound (capsular polysaccharides) and act as potential lectin-reactive site [35]. Owing to the ability of lectins to form complexes with microbial glycoconjugates, they can play a key role in the recognition of microbes and thus exert antimicrobial activity.

P. duclauxii and *P. proteolyticum* lectins inhibited Gram-negative bacteria, whereas *P. griseoroseum* lectin inhibited Gram-positive bacteria. *P. proteo*-

6	Glycan specificity*	E. coli	P. aeruginosa	S. aureus	B. cereus
Sample		Zone of inhibition (mm)			
<i>P. duclauxii</i> lectin (50 μg)	D-Ribose, L-Rhamnose, L-Fucose, D-Glucose, D-Arabinose, D-Galactose, D-Fructose, D-Mannitol, D-Lactose, Inulin, Chondroitin-6-sulphate, D-Glucosamine hydrochloride, D-Galactosamine hydrochloride, D-Glucuronic acid, D-Galacturonic acid, N-Acetyl-D-Galactosamine, 2-Deoxy-D-Ribose, Thiogalactoside, Bovine submaxillary mucin, Porcine stomach mucin, Fetuin, Asialofetuin, N-Acetyl neuraminic acid	38±0.07	35±0.13	15±0.24	NS
P. proteolyticum lectin (50 μg)	L-Fucose, D-Galactose, D-Lactose, Chondroitin- 6-sulphate, D-Glucosamine hydrochloride, D-Galactosamine hydrochloride, N-Acetyl-D- Galactosamine, Thiogalactoside, Bovine submaxillary mucin, Porcine stomach mucin, Fetuin, Asialofetuin, γ-globulin, N-Acetyl neuraminic acid	27 ± 0.88	22 ± 0.19	NS	NS
P. griseoroseum lectin (50 μg)	D-Ribose, L-Rhamnose, Xylose, D-Glucose, D-Mannose, D-arabinose, L-Arabinose, D-Galactose, D-Fructose, D-Mannitol, D-Sucrose, D-Maltose, D-Lactose, Melibiose, D-Trehalose dihydrate, D-Raffinose, Maltotriose, Inulin, Pullulan, Starch, Dextran, Inositol, Meso-inositol, Chondroitin-6- sulphate, N-Acetyl-D-Galactosamine, N-Acetyl-D- Glucosamine, 2-Deoxy-D-Glucose, 2-Deoxy-D-Ribose, Thiogalactoside, Bovine submaxillary mucin, Porcine stomach mucin, Fetuin, Asialofetuin, γ-globulin	NS	NS	27±0.39	28±0.43
Ciprofloxacin (10 µg)	NA	40±0.009	40±0.004	27±0.014	32±0.018
Gentamicin (50 µg)	NA	30±0.011	30±0.009	25±0.024	27±0.007

Table 3. Antimicrobial activities of purified Penicillium sp. lectins.

*Glycan specificity of lectins from Penicillium sp. [12]

NA: Not applicable

NS: Zone of inhibition not shown by lectins

Table 4. Minimum inhibit	ory concentrations of purified	<i>Penicillium</i> sp. lectins.

Test organism	Minimum inhibitory concentration (µg)			
	P. duclauxii lectin	P. proteolyticum lectin	P. griseoroseum lectin	
Escherichia coli	7.5	15	NS	
Pseudomonas aeruginosa	10	20	NS	
Staphylococcus aureus	45	NS	22.5	
Bacillus cereus	NS	NS	17.5	

NS: Zone of inhibition not shown by lectins.

lyticum and *P. griseoroseum* lectins exhibited strong selective antibacterial activity. Interactions of lectin with lipopolysaccharides present in the cell wall of Gram-negative bacteria could be responsible for its antibacterial action [50]. Lectins could also interact with N-acetyl-D-glucosamine, N-acetylmuramic acid and tetrapeptides linked to N-acetylmuramic acid and present in the cell wall of Gram-positive bacteria [50]. *P. duclauxii* [40] and *P. proteolyticum* [41] lectins are nonspecific towards N-acetyl-D-glucosamine. Thus, their non-inhibitory action towards Gram-positive bacteria (which possess high levels of N-acetyl-Dglucosamine in their cell wall) further corroborates this. However, *P. griseoroseum* lectin exhibits specificity towards N-acetyl-D-glucosamine [12] and inhibits Gram-positive bacteria, which substantiates lectinglycan interaction leading to bacterial inhibition. In the case of *S. aureus*, *P. duclauxii* lectin might form a channel on the cell membrane resulting in an outflow of cellular contents, thus leading to cell death. The thicker murein layer in the case of *Bacillus cereus* probably acts as a barrier in *P. duclauxii* and *P. proteolyticum* lectins and thus does not exhibit any inhibitory action.

Crude lectin extracts from *P. corylophilum*, *P. purpurogenum* and *P. expansum* exhibited both antifungal and antibacterial activities [39]. *A. panamensis* [38] and *A. gorakhpurensis* [37] purified lectins also displayed a considerable antibacterial potential. Endophytic fungal lectins from *A. flavus*, *F. moniliforme* and *F. oxysporum* exhibit nonselective antibacterial activity towards *K. pneumonia*, *P. mirabilis*, *S. marcescens* and *S. aureus* [51].

Although many microbial lectins have been characterized, only a few showed antifungal effects [52,53]. Lectins having specificity towards chitin can inhibit fungal growth as the fungal cell wall is composed of chitin (polymer of β -(1, 4)-N-acetyl-D-glucosamine) [50]. However, purified *P. duclauxii* [40] and *P. proteolyticum* [41] lectins are nonspecific towards Nacetyl-D-glucosamine and thus does not exhibit any antifungal activity.

Owing to the potential antioxidant and antibacterial actions of *Penicillium* sp. lectins, these compounds could have prospective biomedical applications and may be used further for microbiology studies.

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