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Chitosans from *Rhizopus stolonifer* (strain CBMAI 1551): Characterization and Dense Film Formation

Douglas Henrique Cardoso[ac], Thiago Ferreira da Conceição[b], Admir José Giachini[a], Márcio José Rossi[a]

[a]Departamento de Microbiologia, Imunologia e Parasitologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, CP 476, 88040-900, Florianópolis, SC, Brazil

[b]Departamento de Química, Centro de Ciências Físicas e Matemáticas, Universidade Federal de Santa Catarina, CP 476, 88040-900, Florianópolis, SC, Brazil

[c]Corresponding author at: adress as [a]; Tel: +55 48 3721 5204; Fax: +55 48 3721 2011; *E-mail*: douglasbiotec@hotmail.com; Orcid ID:orcid.org/0000-0002-7046-6186

ABSTRACT

Chitosan is a bioactive amino polymer with wide applications. Mainly derived from chitin of marine sources, its traditional production still has some drawbacks such as irregular supply, low quality of product and lack of standardization. Farther, extraction processes are time-consuming with considerable environmental impacts, an extremely non-green process. Many works have shown the possibility of producingnative chitosan from Mucorales fungi, which is more easily extracted. Such process is advantageous due to low costs, process control and great possibility of high quality products. Moreover, the extraction of chitosan is faster and generates less pollutants. In this scenario, the possibility of standardized production allied with facilitated extraction and less probability of toxicological side effects from marine sources are characteristics that motivated this work. A Mucoralesisolate was cultured and the chitosans – native and semi-synthetic – obtained through heterogeneous extraction were compared. Results show substantial differences between them. Those differences are related to the processes required for extraction, yield, productivity, and quality. This work reinforces that Mucorales fungi excel as an alternative for chitosan production.

Indexing terms/Keywords

fungi, chitosan, deacetylation, Mucorales

Academic Discipline And Sub-Disciplines

Biotechnology, Bioprocesses, Polymer Science, Natural Products, Mycology

SUBJECT CLASSIFICATION

Biotechnology, Mycology

TYPE (METHOD/APPROACH)

Bioprocess development, natural polymers extraction, polymer characterization

INTRODUCTION

Chitosan is a semi-synthetic polymer mainly derived from the thermochemical deacetylation of marine derived chitin. Deacetylation transforms chitinin a more easily soluble polymer, chitosan, with broad applications. Chitosan is a nontoxic, film-forming, antioxidant, antimicrobial, biocompatible, mucoadhesive polymer, which presents a unique polycationic charge between natural polysaccharides [4], [13], [19], [33].

Chitosan is a versatile material capable of forming films, membranes, blends, capsules, and gels, with applications in many areas, with the advantage of being biodegradable [4], [33]. Many works have focused on identifying alternatives for the traditional methods of chitosan obtainment to overcome the unstable supply due to climate instabilities, absence of quality control during production, but especially in the search of a standardized means of obtainment and processing[2], [6], [18], [22], [31], [32], [36]. Fungi of different groups are interesting options for this purpose, especially with the hope to achieve a standardized product, more appropriated for pharmaceutical and medical purposes [13], [18].

Of the many options, one group reveals special attributes and is attractive: the moulds in theOrder Mucorales,Subphylum Mucoromycotina (ex-Zygomycetes). Mucorales is represented by rapid growing moulds, often non-producers of mycotoxins, and by showing constitutive chitosan in all their life cycle, which can be more easily extracted [2], [13], [35]. A specimen of this group was specifically isolated for this study and identified.

Thermochemical processes were used for extraction and deacetylation of chitosans[34], but no further purification was employed at this stage. Attention was made for the detection of native chitosan, which was treated separately from the chitosan obtained by deacetylation of chitin. In a preliminary attempt to verify the aptitude of these chitosans for applications, dense films were produced [5] and were described qualitatively. Commercial chitosans were compared with the chitosans obtained. Results of yield, productivity, deacetylation degree, purity and film-forming capacity are discussed.

The aim of this work was to characterize the chitosans from one isolate as the first step for a bioprocess development.

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MATERIAL AND METHODS

Chemicals

Crab chitosan (purity 99%) was obtained from Sigma Aldrich™ (CTSs) and a crustacean chitosan from a commercial producer (purity not specified) (CTSc) and used without any further modification. Glicosamine.HCl (GlcN.HCl) (purity 99%) was obtained from Sigma Aldrich™. All other chemicals were of analytical grade.

Strain

A Mucorales strain was isolated from commercial strawberries and maintained in diluted Czapek Dox medium (CD) [15], at 25 °C in the Bioprocess Laboratory, UFSC/SC, Brazil. For long-term preservation, spores were cryopreserved in glycerol 80% in a -80 °C freezer [25]. The strain was deposited in the culture collection CBMAI/Unicamp (ColeçãoBrasileira de Microorganimos do Ambiente e Indústria), under the code CBMAI 1551 (1551).

Culture media

For morphological identification the following mediawere used: CYA (Czapek yeast extract agar), MEA (Malt extract agar) and G25N (25% glycerol nitrate agar). These media weresterilized for 15 min in an autoclave and distributed in Petri dishes (∅ 90 mm for all media, but ∅ 50 mm for CYA at 5 °C) [25].

For submerged culture of the strain, we used YPG broth [34] with minor modifications, as follows (in g L^{-1}): glucose 20.0, peptone 10.0, yeast extract 1.0, $(NH_4)_2SO_4$ 4.8, $MgSO_4.7H_20$ 0.5, $CaCl_2$ 0.2 and 0.25 mL L^{-1} of PPG (polypropyleneglycol) anti-foam, pH 4.5, adjusted with buffer citric acid/sodium citrate 0.45 mol L^{-1}. All material and ingredients were sterilized by autoclaving (15 min at 121 ºC). Inoculum was made by spores (10^6.mL^{-1}), obtained in YPG agar from Roux bottles [20]. Cultures were made in 1 L flasks adapted with an aeration system, as described: aeration was made with a 0.33 vvm air flow, measured in a flowmeter (Vögtlin Instruments GmbH,Aesch, Switzerland), in a range of 1.6 – 16.0 Nl.h^{-1}. The air was flown through a chamber with a glass wool and water for retention of solids and humidification of air, and through a 0.22 µm PTFE membrane filter (Milipore™) to avoid contamination. Flasks were maintained at 25 °C for 4 days in a BOD.

Strain identification

The isolate was morphologically identified by a plate regimen [25] and microscopic mounts. The strain was growth in three different media at three different temperatures (5, 25 and 37 °C for CYA plates, and 25 °C for MEA and G25N) in Petri dishes, and colonies evaluated after 7 days.

Chitosan extraction

Extraction, deacetylation and precipitation of chitosans were conducted as described byTrutnau at al. [34] with smallmodifications, as follows: the acid solution after demineralization was reserved for precipitation of native chitosan (not deacetylated), which was compared with the semi-synthetic chitosan (obtained by deacetylation). Obtained chitosans were identified as: CTS1551ss(semi-synthetic) and CTS1551n (native).

Total amino sugars estimation

The method of MBTH (3-methyl-2-benzothiozolone-hydrazone-hydrochloride) was conductedaccording toZamani et al. [39], except that only the total amino sugar presented was quantified. A linear correlation (R^2=0.9987) of absorbance (at 650 nm) with glucosamine.HCl monomers (0 to 100 mg L^{-1}) was conducted in the same conditions of the samples, and used as standard. The absorbance was read in a spectrophotometer (BEL Photonics, model 1105). Results reflect the media and standard deviation of triplicates.

Conductometric titration

All samples were subjected to conductometric titrations to estimate the deacetylation degree. Briefly, samples (200 mg) were dissolved in 40 mL HCl 54 mM and stirred at 500 rpm for 18 h at 25 °C, then 0.5 mL NaOH 165 mM were added every 20 s as described by Alvarenga et al.[1].The assays were performed using a thermal magnetic stirrer (Fisatom, model 752) and a thermal conductometer (Tecnal, model TEC-4MP). Results reflect the media and standard deviation of triplicates.

Fourier transform infrared spectroscopy (FTIR)

Analysis were run in a Perkin-Elmer PC-16 spectrophotometer with a resolution of 2 cm^{-1} in the range of 400–4000 cm^{-1}[40], using KBr pellets. An average of 32 scans were recorded for each sample; The analyses were performed only for samples CTSs, CTS1551ss, and CTS1551n.

Dense film preparation

To estimate the applicability of chitosans obtained for biotechnology purposes, they were evaluated for film-forming capacity by production of dense films as presented by Beppu et al. [5]. The films obtained were named as follows: F1551ss (semi-synthetic chitosan from strain 1551),F1551n (native chitosan from strain 1551), Fs (chitosan from Sigma Aldrich®) and Fc (chitosan from a commercial producer).

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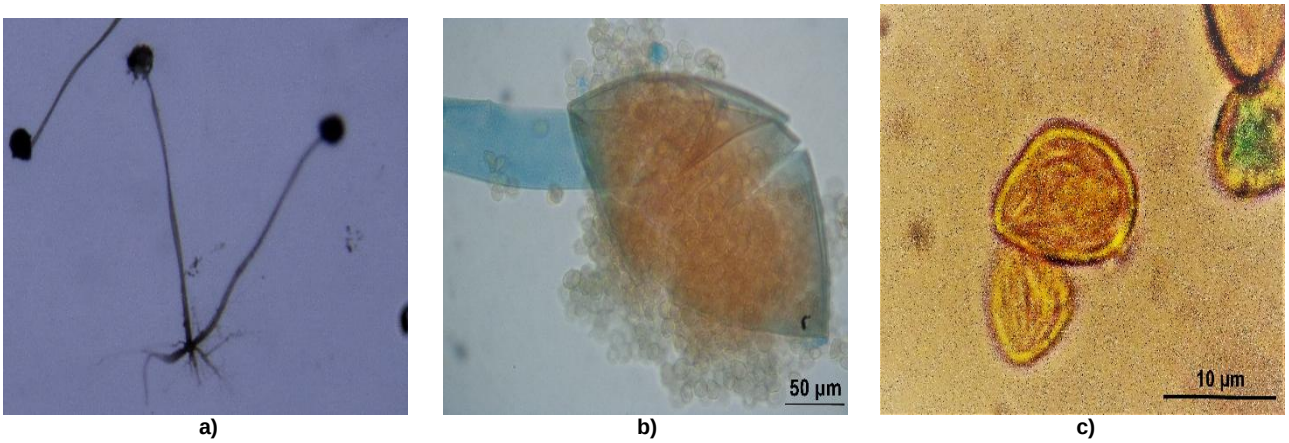
RESULTS AND DISCUSSION

Morphological identification of strain 1551

Microscopic observations of the mycelium and colonies were made for morphological identification. The hyaline mycelium has a wooly appearance, presents large non-septated hyphae, and rapidly produces sporangiophores. The plate regimen showed no zygospore formation; plates at 5 and 37 °C had no apparent growth; growth was observed in all plates at 25 °C with increasing intensity in the following order: CYA>MEA>G25N; sporangium abundance followed the same order; it was observed abundant formation of stolons between hyphae; columellae derived from sporangia collapsed in umbrella shapes; and spores had striated walls when seem by an optical microcopy (100x), as can be seen in Figure 1. These characteristics as per Pitt and Hoching[25] identify this isolate as belonging to the genus*Rhizopus*, species *stolonifer*.



a) b) c)

Figure 1 Rhizopus stolonifer, strain CBMAI 1551. a) Sporangiophores showing non-ramified sporangia and rhizoids (Transillumination, 64x, Zeiss Stereomicroscope Stemi 2000-C); b) An umbrella shaped sporangium after release of sporangiospores (Lactophenol cotton blue mount, 400x, Zeiss Primo Star Microscope with AxioCam ERc5s Color Microscope Camera); c) Striated sporangiospores (Oil immersion, 1000x, Zeiss Primo Star Microscope with AxioCam ERc5s Color Microscope Camera).

Chitosans obtained

Native chitosan (CTS1551n) was precipitated from the acid solution in the second step of extraction, while chitosans obtained by deacetylation (CTS1551ss) wereprecipitated after deacetylation in the fourth step. Results of concentration and yield are presented in Table 1. Chitosans were quantified as dry weight (DW) and compared with theliterature.

Table 1 Concentration (C_{CTS}) and Yield (Y_{CTS}) of chitosans from Rhizopus stolonifer.

Source	Type of Chitosans[a]	C_{CTS}(g L^{-1})	Y_{CTS}(%)[b]	References
R. stolonifer	SS	0.282	7.76	This work
R. stolonifer	N	0.254	6.24	This work
R. stolonifer	N	0.102	2.25	[32]
R. stolonifer	N	0.070	1.90	[17]

[a]Type of obtainment of chitosans: SS = semi-synthetic chitosan (via deacetylation of chitin); N = native chitosan (natural, not deacetylated).

[b] Grams of chitosan per 100 grams of biomass.

Strain 1551 produced 0.254 g L^{-1} of native chitosan and 0.282 g L^{-1} of semi-synthetic chitosan in a total of 0.536 g L^{-1}, which represents a 14.0% yield on DW basis. Although yield of native chitosan forstrain 1551 was from 2.8-3.3 times greater than other studies [17] and [32],it must be noted that different methods of extraction, medium composition, incubation conditions, and different strains were employed for each study. When compared with another Mucorales member(*Mucorrouxii*),for example, even with the same conditions of incubation and methodology of extraction, yields are far beyond(between 0.8-0.9 % of total chitosanyield - semi-synthetic plus native) [34]. It's important to note that yield is not only directly related to these variables, but also by the species and strain. Usually, for practical purposes, thermo chemical methods (high temperature, high concentration of alkalis) are employed, although this harsh process can diminishes yield, especially by degradation of fibers [4]. Therefore, strain 1551 was not been subjected to any optimization in chitosan production yet.

Deacetylation degree (DD)

Results from conductometric titrations are summarized in Table 2.

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Table 2 Conductometric titration for the determination of deacetylation degree of chitosans.

Samples	DD[1] (%)	Informed DD (%)
CTSs	84.1 ± 3.2	75.0-85.0
CTSc	66.3 ± 9.4	85.9
CTS1551n	77.3 ± 3.1	This work
CTS1551ss	75.6 ± 3.6	This work

[1]Mean and standard deviation of triplicates.

Results for CTSs, a semisynthetic crab chitosan from Sigma-Aldrich, were in the range as informed by the manufacturer, but on the contrary, deacetylation degree of CTScwas consistently below the degree informed. The DD of CTS1551n (77.3 ± 3.1) was lightly above of that of CTS1551ss, which was subjected to a deacetylation step. In a Apreviuosly work with *R. stolonifero*btained native chitosan with 60,9% of deacetylation by infrared analysis [17]. *Mucorrouxii* had both chitosans treated as one and the DD obtained was higher (86-88%) [34], but it should be noted that, as a Mucorales, *M. rouxxi* produces natural chitosan, which, subjected to a deacetylation step, enhances the overall DD. It should be noted that although this can provide a higher final chitosan yield, the process also generates a more heterogenic product, as native chitosan crystallinity, viscosity, molecular range distribution, among other characteristics, are changed when thermo chemical deacetylation, an unquestionably severe process, is employed. Values of 86.1, 85.2 and85.6 % DD were found for *A. coerulea, M. rouxii* and *Rhizopus oryzae,* respectively, under submerged cultivation[35]. The easily extracted chitosan from *R. arrhizus* was determinedto be 86 % DD in a study with agro industrial residues [6]. Fungalchitosans have some distinctive features as absence of allergenic shrimp-related proteins and minerals, among others substances, as stated by Dhillon et al.[13] and Kaur &Dhillon[18]. In spite of the great interest in increasing the final chitosan yield forthe fungi used as alternative for its obtainment, many researches havefocused on the native or direct extracted chitosan ([2], [13], [18], [35], [36]) instead of the semi-synthetic form, via deacetylation of chitin. In addition to being easilyextracted, what means less costs and time of production, there is a great chance of finding a way to achieve a native chitosan with high deacetylation degree, and with regular medium-low molecular weight fibers and minimal degradation of fibers. These features are mostly desired because they can open a wide spectrum of applications in areas of pharmaceutical or biomedical purposes.

Amino sugars

The percentage of total amino sugars detected in the samples are presented in Table 3.

Table 3 Percentage of total amino sugar recovered from chitosan samples.

Samples	Mean[a] (%)	SD[b]
CTS1551ss	43.1	±2.27
CTS1551n	63.9	±1.79
CTSs	76.1	±1.04
CTSc	60.0	±1.87

[a] Mean of triplicates.

[b] Standard deviation.

The percentage of total amino sugars recovered was below of thatobtained by Wang andco-works [35] with *A. coerulea, M. rouxii*and*R. oryzae*, which all had a purity above 80% (86.1, 85.2 and85.6, respectively). Variations on total amino sugars are related to many factors such as the species in study, the type and time of cultivation, and the methods employed for extraction [2]. Although important, the search for optimal conditions was not a subject of the present study. It is necessary to note that none of the chitosans in this work were subjected to any further purification. Discoloration of chitosan with NaOCl, H_2O_2, $NaClO_2$, $KMnO_4$[12], ozone [28] or ultraviolet radiation [38] usually diminish yield, but improve purity and quality. This is extremely noteworthy, especially for biomedical applications. Although not employed in this work, this step will be prioritized in future studies.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of chitosans obtained from strain 1551 are compared with a standard CTSs as in Fig.2.

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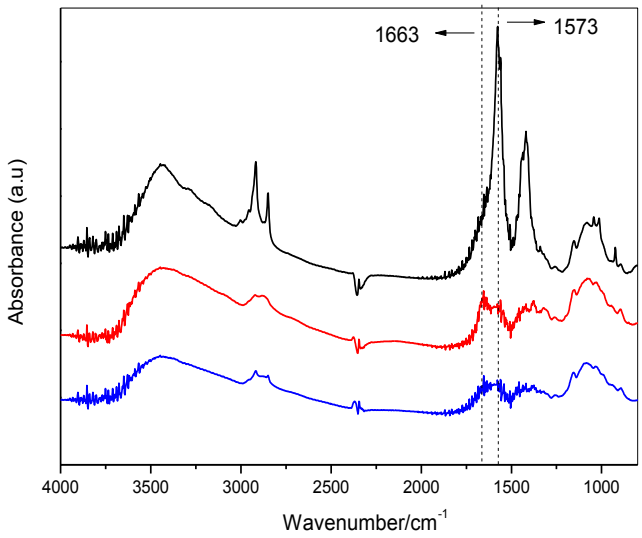

Figure 2 FTIR spectra for chitosans.Standard CTSs (—), CTS1551n (—), CTS1551ss (—).

The spectra for all samples show the typical absorption bands of chitosan: a broad band in the range of 3000 cm^{-1} to 3700 cm^{-1}, related to O-H stretching, two bands in the range of 2800 cm^{-1} to 3000 cm^{-1}, related to C-H stretching, one at 1663 cm^{-1}, related to C=O stretching of the amide group (acetylated units), one at 1573 cm^{-1}, related to N-H bending, one at 1420 cm^{-1}, related to C-H bending, and a series of other absorption bands below 1500 cm^{-1} related to other functional groups[16], [21], [24].It can be observed in Fig. 2 that, for CTS1551ss, theabsorbance of the amine group (1573 cm^{-1}) is much greater than that of acetylated groups (1663 cm^{-1}), indicating a higher deacetylation degree. For the standard CTS, the absorbance of the amide group is more intense, whereas for CTS1551n both absorbances are of similar intensities. This result indicates that CTS1551ss has the highest deacetylation degree among the analysed samples.

Dense films

To evaluate the film-forming potential of chitosans, simple dense films were produced (Fig.3).

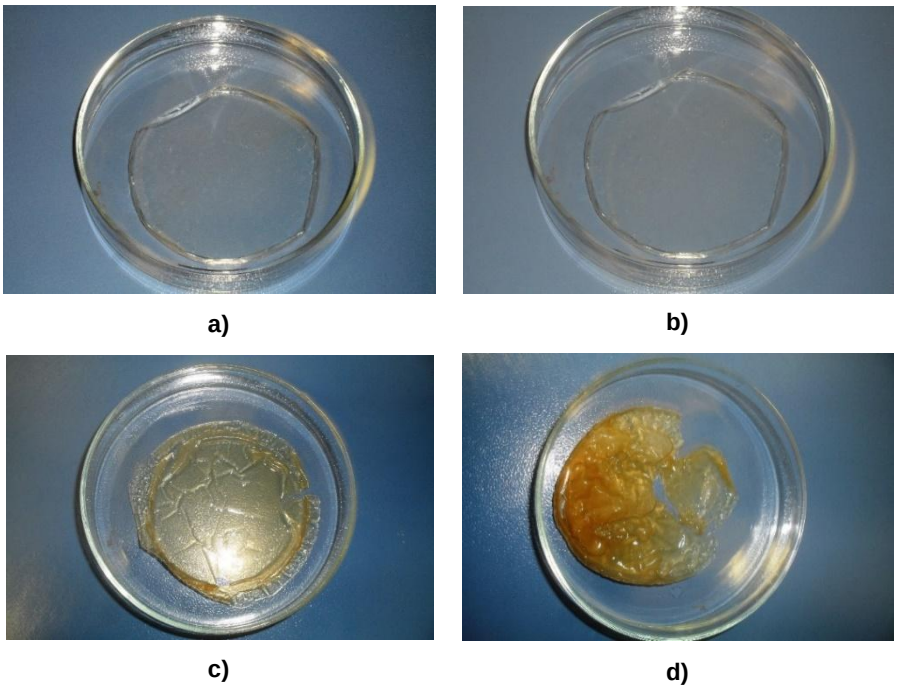

Figure 3 Aspect of dense films from different sources. Fs, film done with chitosan from crabs (Sigma Aldrich) (a); Fc, filmofthe commercial chitosan from crustaceans (non-identified producer) (b); F1551ss, filmof the semi-synthetic chitosan from strain 1551 (c); F1551n, film of the native chitosan from strain 1551 (d).

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This preliminary study shows remarkably differences among the sources used, as shown in Figure 3. As expected, Ms hada rapid coagulation, and after the necessarywashings, a hyaline, translucent, flexible, and manageable film was formed. The Mc from the other commercial source also shown good film-forming capability, but the film had a yellow coloration, which probably reveals a not fully purified material. Some attempts were made to decolorizethe chitosans obtained from strain 1551 [34]with alcohol and acetone, and with cold sodium hypochlorite[23], but they were all unsuccessful. This may have affected quality and yellowish appearance of the films. Although these are preliminary results, as far as we know few studies have focus on film-forming capability of fungal chitosans [29].

Table 5Qualitative evaluation of chitosan films.

Films[a]	Description
Fs	Hyaline, translucent, flexible, manageable
Fc	Light yellow, translucent, flexible, manageable
F1551ss	Dark yellow, translucent, flexible, manageable and wrinkled
F1551n	Dark yellow, translucent, flexible, fragile and wrinkled

[a] abbreviations as in Figure 3.

Results, taken as a whole, evidence some differences between the chitosansobtained. The chosen of an alternative technique for chitosan production has to reckon these differences to decide which one could be more interesting, not only considering the more green friendly processes, when compared with the traditional crustacean sources, but also take into account results of yield, quality of the products obtained, time of production, process control, and, of course, the real possibility of obtaininga standardized product.

CONCLUSIONS

The plate regimen method has proved to be easy, cheap, fast and robust for the identification of a fungalisolate when molecular tools are not available. Although results of the deacetylation degree from conductometric titration and FTIR are contradictory, the FTIR spectra of CTS1551n is quite similar to that ofstandardized CTSs, which is around 84% deacetylated. A more specific method like NMR will be employed in a full characterization for next studies. Even not fully purified, the total amino sugars contentsforCTS1551n washigher than the crustacean chitosan CTSc. The films formed from fungal chitosans were wrinkled, but this may be dueto their raw state.Strain 1551 is fast growing, has high yields of easily extracted chitosans, and should now be subjected to cultivation in a bioreactor, to obtaing data on biomass production(yield) and chitosan DD levels. The search for an efficient and specific technic for the bleaching of fungal chitosans should be a priority.

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