Production and Purication of Antifungal Compound from Weisselaparamesenteroides

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Abstract

The production for antifungal compound was done in MRS broth(De Man rogosa & sharpals medium) .The Antifungal compound was partially purified using ammonium sulphate precipitation followed by column chromatography. The relative molecular weight of purified antifungal compound was checked using SDS-PAGE, found to be between 3KD to 5KD. From IR analysis, the functional groups were determined which may be hydrogen bond, chelated hydrogen bond, alkenes, aromatic alkenes, nitro group, alcohol, phenol, ester, ether or halo-alkenes. The compound was maximally absorbed at the wavelength of 212 nm and 225 nm in UV absorption spectrophotometer. The purified enzyme was quite stable at 40°C and pH range 5.2-6.8. The antifungal activity of the compound was lost after treatment with proteases.
Conclusions: The purified antifungal compound is a low molecular weight protein and may be acyclic heteroannulardienes. We report for the first time antifungals from Weisselaparamesentroides of vegetable origin.Antifungals from the Weisselaparamesentroides can be exploited as biopreservative to prevent post harvest fungal spoilage of agricultural produce.
KEYWORDS- Antifungal, biopreservative, Weisselaparamesentroides, spoilage fungi, lactic acid bacteria.

I INTRODUCTION

Moulds and Yeasts are commonly involved in the post harvest spoilage of vegetables. Post harvest spoilage of vegetables can be minimized either by maintaining hygienic conditions or using chemical preservatives. Now a day’s consumer demand is increasing for use of ecofriendly biopreservatives instead of chemical preservatives due to their health hazards.

Use of LAB in food preservation is known from ancient times. LAB are found on plants and generally recognized as safe, so they can be alternative in biopreservation of vegetables. There are reports on production of antifungal compounds by LAB. They are found to produce many antimicrobial products like organic acids, bacteriocins, antibiotics other products like ethanol, hydrogen peroxide, carbon dioxide, diacetyl and acetaldehyde.

In order to exploit the antifungal potential of lactic acid bacteria it is essential to study the factors affecting production of bioactive compound. However, the published reports in this area are rather scanty. Lactic acid bacteria grow only in complex media and are therefore considered fastidious in their nutrient requirements. Many of the genes encoding for anabolic enzymes in LAB are present on plasmids, which may be easily lost during growth in the absence of appropriate pressure. Therefore, in general, the concentration of nutrients required by a strain depends on i) the source from which the strain was isolated, ii) period of dormancy and iii) medium used to maintain the strain. This adds to tediousness of developing a generally applicable defined medium for these organisms.

In our previous study wehave found that, some LAB isolates from vegetables produced antifungal substances. One of the isolate Weisselaparamesentroides from

spinach exhibiting strong antifungal activity has been used in current study (Satheet al. 2007). Further, Weisselaparamesentroides was grown for 72 h in MRS broth and antifungal substances were recovered by ammonium sulphate precipitation. The dialyzed compound was further purified by Sephadex column chromatography. The purity of the compound was assessed by SDS gel electrophoresis. An antifungal activity of the bands on gel was determined by overlaying soft malt extract containing the spore suspension of the target fungi. Structural analysis was performed by using UV and IR spectra.

II MATERIAL AND METHODS

Studies on production optimization of antifungal activity

Production of antifungal activity in Weisselaparamesentroides was carried out at in MRS broth. All incubations were under microaerobic environment unless specified. The sensitive fungal culture, Rhizopusstolonifer was used as target pathogen for determination of antifungal activity. Production of antifungal substance in liquid medium by Weisselaparamesentroides and antifungal activity assay were performed as per the protocol described elsewhere (Satheet al. 2007).The concentration of antifungal compound per milliliter of cell free supernatant (CFS) was determined by agar well diffusion method in terms of arbitrary units (AU) defined as the reciprocal of highest dilution at which fungus was inhibited (Wan et al. 1995).

Ammonium sulphate precipitation

The proteins present in culture supernatant were precipitated by slow addition of solid ammonium sulphate to 40%, 60% and 80% saturation; separately at 4ºC with continuous stirring. The mixture was incubated at 4°C for 12 h. Then it was centrifuged at 10,000 rpm for 15 min. at 4ºC. The precipitate was recovered in 10 mM phosphate buffer (pH 6.5). This procedure was followed for obtaining precipitate from 40%, 60% and 80% ammonium sulphate saturated solution; separately.

Partially purified compound and culture supernatant were tested for antifungal activity and calculated in terms of Arbitrary Unit per ml as described in Satheet al. (2007).Protein concentration of culture supernatant and partially purified compound was determined by Folin-Lowry's method (Lowry et al. 1951).

Dialysis of partially purified compound

Partially purified antifungal compound was prepared as described by Bauer et al. (2003) with some modification. The ammonium sulphate precipitate was dissolved in 10 mM phosphate buffer of pH 6.5 and dialyzed against the same buffer by using 3 KD molecular weight cutoff size dialysis membrane. Dialysis membrane was activated by treatment with 100 mM $NaHCO_3$ and 10 mM EDTA. Then it was boiled for 10 minutes followed by washing with distilled water twice. The membrane was stored in 4% chloroform. The dialyzed sample was stirred for 24h at 4ºC by changing the phosphate buffer 3 times within 24h to remove fatty acids, including residual Tween 80. The protein content and antifungal activity of dialyzed sample were determined.

Column chromatography

Purification of the partially purified compound was carried out by column chromatography using Sephadex G75 having fractionation range 3 – 100 x 10^{-3}. The column was cleaned using water and rinsed with acetone. After drying the column a small piece of cotton was placed at its bottom. Sephadex G75 gel was then packed in the column by using distilled water as solvent system. The crude antifungal compound was loaded at the top of the column and eluted using MilliQ water as solvent system. Fractions were collected, where flow rate was adjusted to 1 ml per

minute. The ultraviolet absorption spectrum at 280nm and activity of each fraction was detected.

SDS-PAGE electrophoresis

Molecular weight of antifungal compound present in active fractions was determined by SDS-Polyacrylamide gel electrophoresis using 12% polyacrylamide followed by silver staining (Merrilet al. 1983).

Infrared and Ultraviolet spectroscopy

Thepurified sample was kept at 40ºC for drying and the powder obtained was subjected to infrared spectroscopic analysis for determination of functional groups. An UV absorption maximum of liquid dialyzed sample was also checked by UV spectrophotometer.

Characterization of the Antifungal Compound

Sensitivity to temperature

The effect of temperature on activity of the antifungal compound was determined by dispensing 1 ml of purified antifungal compound dissolved in 10mM phosphate buffer (pH6.5) in screw capped bottles and treating at a temperature of 40ºC, 60ºC, 80ºC, and 100ºC for 10 min in water bath. The antifungal solutions were cooled at 4ºC and residual antifungal activity was determined against target microorganism Rhizopusstolonifer by using agar well diffusion method. The purified antifungal solution was also subjected to autoclaving temperature i.e. 121ºC for 15 minutes.

Sensitivity to pH

To determine the effect of pH on activity of the compound, it was recovered in 1 ml of 10 mM phosphate buffer of varied pH (pH 5.7-8) in various tubes incubated for one hour at 28ºC and the residual antifungal activity in each tube was determined against target organism Rhizopusstolonifer after adjusting pH to neutral.

Sensitivity to enzymes

The sensitivity of antifungal compound was determined by enzymes Proteinase K, Pepsin, Papain and Trypsin. All enzymes were obtained from Sigma Chemical Company USA and were dissolved in 10mM phosphate buffer (pH6.5) at concentration 1mg/ml.100µl of antifungal solution was mixed with 100µl of enzyme solution and incubated at 37ºC for 1 hr. The antifungal compound solution without any enzymes served as control (Munimbazi and Bullerman, 1998).The residual antifungal activity of treated sample was tested by agar well diffusion method against Rhizopusstolonifer as target organism.

Storage stability of antifungal compound

The storage stability of the purified anti fungal compound was determined by incubating the compound at -20, 4 and 28ºC for six month. The antifungal activity of sample was tested monthly by agar well diffusion method against Rhizopusstolonifer as target fungi.

Spectrum of antifungal activity

Antifungal activity spectrum of culture supernatant and purified antifungal compound was determined by agar well diffusion method as described in Satheet al. (2007). Activity was determined in terms of zone of inhibition (mm).Activity spectrum of culture supernatant and partially purified antifungal compound was determined against Aspergillusflavus, RhizopusStolonifer, SclerotiniumOryzae, Fusariumgraminearum, Rhizoctoniasolani, Aspergillusphonesis.

III RESULTS

Effect of medium type on production of antifungal compound

Weisselaparamesentroidesexhibited (1280 AU ml^{-1}) antifungal activity against Rhizopusstolonifer when grown in MRS medium.

Ammonium sulphate precipitation

The antifungal compound was precipitated maximum at 60% saturation of ammonium sulphate . The precipitate was obtained by centrifuging the ammonium sulphate saturation at 10000rpm for 10 min was dissolved in 10 mM phosphate buffer (pH 6.5). The total activity and total protein content were estimated to determine the specific activity, fold purification and percentage yield (Table 1). After 48h of fermentation the protein content of original culture supernatant was 2.6 mg ml^{-1} and the antifungal activity was 1280 Au ml^{-1}. At 60% saturation of salt, the compound was 5.2 fold purified with 10% yield. The yield decreases due to the loss of the antifungal compound during purification.

Dialysis of the precipitated compound

The salt present in partially purified compound was removed by dialysis using 3 KD cutoff size dialysis membranes (Sigma-Aldrich, USA). Dialysis was done overnight with continuous stirring at 4°C. After dialysis, the activity and percent yield decreases indicate that the other low molecular weight antifungal compounds present (> 3 KD) may be passed through the dialysis membrane (3 KD cutoff size).

Column chromatography

Further purification of partially purified compound was done by column chromatography using Sephadex G75. The flow rate was adjusted (1ml per minute). Twenty different fractions were collected after loading of crude compound at the top of column, which was eluted with distilled water as solvent system. The absorbance of each fraction was measured at 280 nm. The activity of each fraction was checked by Agar well diffusion assay against Rhizopusstoloniferas target fungi. Amongst all these 20 fractions, fraction 4, 5, and 6 showed activity.

SDS-PAGE electrophoresis

Fraction obtained from Sephadex G 75 coloumn chromatography as well as dialysed precipitate were run on SDS-PAGE along with the molecular weight markers ranging from 3KD to 97.4KD. The molecular weight of purified antifungal compound is in between 3KD to 5KD.

Infrared and UV spectroscopy

The purified sample was kept at 40ºC for drying and IR spectrum of the powder obtained was checked for determination of functional groups of the compound. From this IR analysis, the functional groups were determined these may be hydrogen bond, chelated hydrogen bond, alkenes, aromatic alkenes, nitro group, alcohol, phenol, ester, ether or halo-alkenes from the frequencies obtained 3288.4, 2970.2, 1643.2, 1527.5, 1417.6, 1091.6, 615.2, respectively in IR spectrum. (Fig. 1) The compound was maximally absorbed at the wavelength of 212 nm and 225 nm in UV absorption spectrophotometer. Thus the compound may be acyclic heteroannulardienes, which absorbs maximally at 215 nm or it may contain Benzene ring, which absorbs maximally at 229 nm.

Characterization of antifungal compound from Weisselaparamesentroides

Thermal stability of the antifungal compound from Weisselaparamesentroides

Since activity of the antifungal compound was quite stable at 40^oC, experiments were also conducted to see the effect of elevated temperatures on stability of antifungal compound. For this purpose antifungal compound was kept at various temperature (30^oC to 80^oC) for 10 minutes. The antifungal activity was stable up to 80^oC (Table 2).

Effect of pH on stability of the antifungal compound

Stability of the antifungal compound at different pH was checked by pre-incubating the antifungal compound, for one hour, in phosphate buffer at different pH ranging from 5.2-9.0 .The antifungal compound was quite stable within this pH range as tested against Rhizopusstolonifer by agar well diffusion assay. After incubation of antifungal compound in the pH range of 5.2-9.0. Maximum residual activity was observed at pH range 5.2- 6.8 (Table 3)

Effect of enzymes on antifungal activity

The treated sample was tested by agar well diffusion method against Rhizopusstolonifer target organism. The antifungal compound without enzyme treatment showed inhibition zone diameter of 13cm while inhibition zones were not observed after treatment with enzyme pepsin and trypsin. Inhibition zones observed after treatment of Lipase, which shows inhibition diameter of 12 cm (Table 4). This clearly indicates that there was a loss in the antifungal activity of the compound after treatment with pepsin, trypsin and proteinase K but no loss by treatment with lipase, thus indicating that it is a protein in nature and not a lipid.

Storage stability of antifungal compound

The antifungal activity of purified compound was lost during prolonged storage. The activity was stable during storage for three months at 4 and 25°C, but it decreased after three months. Activity could not be recovered after storage for 7 days at –20 °C.

Spectrum of antifungal activity

Antifungal Spectrum of compound from Sephadex G75 columnagainst seven spoilage fungi was determined by welldiffusion method (Table 5). Maximum zone of inhibition was obtained against RhizopusstoloniferandSclerotiniumoryzae.

IV DISCUSSION

Optimum conditions for production of antimicrobials differ even in closely related strains of LAB (Muriana&Luchansky 1993). The important aspects in the study of antimicrobials are their production and purification.

In the present investigation we found that Weisselaparamesentroidesproduce maximum antifungal substance in MRS broth as compared to other media used as found in our previous study.

Okkers et al. (1999) isolated pentosin TV 35 b,abacteriocin like peptide peptide from Lact. pentosus with fungistatic effect on Candida albicans. This peptide was purified by ammonium sulphate precipitation, followed by SP- Sepharosecation exchange chromatography. The molecular weight of the peptide was 2.35- 3.4 kDa. Activity of this peptide was lost after treatment with proteiolytic enzymes and the compound was quite stable over a wide range of pH and temperature.

Yang and Clausen (2005) found that L.casei and Lact. acidophilus together inhibit mould growth in liquid culture. The compounds were not identified but found to be heat resistant and showed antifungal activity after neutralization.
Lavermicocca et al. (2000) found production of antifungal activity by Lact. plantarum and purified antifungals were identified as phenyllactic acid, p-hydroxyphenyllactic acid and palmitic acid
Magnussionet al.(2001); Rouse et al. (2008); Batish et al. (1989); Roy et al. (1996) and Gouramaet al. (1997) reported the production of antifungals by LAB which was due to proteinaceous compound.
We have isolated Lact. plantarumfrom cucumber and it produced protein l compound which is strongly antifungal and showed broad spectrum of activity against vegetable spoilage fungi. Antifungal substance was isolated from the cell free fermented broth by ammonium sulphate precipitation
The antifungal compound lost its activity after its treatment with proteolytic enzymes that indicates its protein nature. It was not inactivated by lipase indicating that the antifungal activity is not due to lipids. The antifungal compound was found to be heat stable and retained its activity even after its exposure up to a temperature of 100°C but activity is lost after autoclaving. Many researchers report similar observations.
Attempt to extract antifungal metabolites was failed antifungal activity is precipitated with ammonium sulphate at 60 % saturation. Partially purified compound showed strong antifungal activity. It is observed that antifungal metabolite is of low molecular weight (3 KD).

Antifungal metabolite was quiet stable up to 80°C and its antifungal activity is reduced after treatment at 100°C. Antifungal compound is active in the pH range of 6.8 to 8.0 and also stable up to three months of storage at 4 and 25°C. Its activity is lost after three months.

Infra Red analysis indicate that functional groups in the compound may be hydrogen bond, chelated hydrogen bond, alkenes, nitro group, alcohol, phenol, ester, ether or halo alkenes. The compound was maximally absorbed at the wavelength of 212 nm and 225 nm in UV absorption spectrophotometer thus the compound may contain acyclic heteroannulardienes or benzene ring.

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REFERENCES

Batish, V.K., Lal, R. and Grover, S., (1989) Screening lactic starter cultures for antifungal activity, Cul Dairy Prod J24, 22–25.

Bauer, R., Chikindas, M.L., and Dicks, L.M.T. (2005) Purification, partial amino acid sequence and mode of action of pediocin PD-1, a bacteriocin produced by Pediococcusdamnosus NCFB 1832. Int J Food Microbiol 101, 17-27.

Drosinos, E. H., Mataragas M., Nasis P., Galiotou M. M. and Metaxopoulos J. (2005) Growth and bacteriocin production kinetics of Leuconostocmesenteroides E131. J ApplMicrobiol99, 1314- 1323.

Gourama, H. and Bullerman, L.B. (1997) Anti-aflatoxigenic activity of Lactobacillus caseipseudoplantarum.Int J Food Microbiol 34, 131-143.

Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A. and Gobbetti, M. (2000) Purification and characterization of novel antifungal compounds from the sourdough Lactobacillus plantarumstrain 21B. Appl Environ Microbiol66, 4084-90.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Radall, R. (1951) Protein measurement with the folin phenol reagent.J BiolChem 193 265-275.

Magnusson, J. (2003) Antifungal activity of lactic acid bacteria, Ph.D. thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Merril, C.R., Goldman, D., and Van Keuren, M.L. (1983) Silver staining methods for polyacrylamide gel electrophoresis.Methods Enzymol 96, 230–239
Moretro T., Aasen, I. M., Storro, I. and Axelsson, L. (2000) Production of sakacin P by Lactobacillus sakei in a completely defined medium.J ApplMicrobio88, 536-545.

Munimbazi, C., Bullerman, L.B. (1998) Isolation and partial characterisation of antifungal metabolites of Bacillus pumilus.J ApplMicrobiol 84, 959-968.

Muriana, P. M. and Luchansky, J. B. (1993) Biochemical methods for the purification of bacteriocins.In Bacteriocins of Lactic Acid Bacteria pp. 41-57.ed. Hoover, D. F. and Steenson L. R. Academic Press. San. Diego, USA.

Okkers, D.J., Dicks, L.M., Silvester, M., Joubert, J.J. and Odendaal, H.J. (1999) Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from Lactobacillus pentosuswith a fungistatic effect on Candida albicans. J ApplMicrobiol87, 726-734.

Rouse, S., Harnett, D., Vaughan A. and van Sinderen, D. (2008) Lactic acid bacteria with potential to eliminate fungal spoilage in foods J ApplMicrobiol104, 915–923

Roy, U., Batish, V.K., Grover, S. and Neelakantan, S. (1996) Production of antifungal substance by Lactococcuslactissubsp. Lactis CHD-28.3.Int J Food Microbiol32, 27–34.

Sathe, S.J., Nawani, N.N., Dhakephalkar, P.K. and Kapadnis, B.P. (2007) Antifungal Lactic Acid Bacteria with potential to prolong shelf-life of fresh vegetables.J ApplMicrobiol103, 2622-2628.

Wan, J., Hicky, M.W. and Coveney, M.J. (1995) Continuous production of bacteriocins, bevicin, nisin and pediocin, using calcium alginate-immobilized bacteria.J ApplBacteriol79, 671–676.

Table 1 Purification of Antifungal Compound from culture supernatant of Lactobacillus plantanum CUK501

Ammonium sulphate saturation (%)	Volume (ml)	Protein (mg ml^{-1})	Total protein (mg)	Activity ($AUml^{-1}$)	Total Activity (AU)	Specific Activity (AU mg^{-1})	Fold Purification	Yield (%)
0	200	2.6	520	1280	256000	432.3	1	100
40	10	0.60	6	1280	12800	2133.33	4.33	5
60	10	1	10	2560	25600	2560	5.20	10
60 after dialysis	10	0.68	6.8	1280	12800	1882.3	3.82	5

Table 2 Thermal stability* of the antifungalcompound from SephadexG75 column.

Exposure Temperature (^oC)	Inhibition zone diameter (mm) against **Rhizopusstolonifer**	**Thermal stability (%)**
30	14	100
40	14	100
50	13	92
60	12	86
70	12	86
80	12	86
100	10	71
120	00	00

*Each data point represents average of triplicate experiments.

Table 3 Effect of pH on stability* of the antifungal* compound from Sephadex G75 column.

pH	Inhibition zone diameter (mm) against **Rhizopusstolonifer**	Stability %
5.2	13	100
5.7	12	92
6.3	12	92
6.8	12	92
7.3	10	77
7.8	10	77
8.3	10	77
9.0	7	54

*Each data point represents average of triplicates.

Table 4 Effect of enzymes on activity* of the antifungal compound from Sephadex G75 column.

Treatment of AFS# with enzymes	Inhibition zone diameter (mm) against **Rhizopusstolonifer**
Pepsin 0	0
Trypsin	00
Proteinase K	00
Papain	00
Lipase 24	12
AFS(control)	13

*Each point represents average of replicates#AFS, Antifungal substance.

Table 5 Antifungal Spectrum* of compound from Sephadex G75 column.

Test fungus	Code number	Antifungal activity*
Aspergillusflavus	MTCC-2192	++
Rhizopusstolonifer	MTCC-1546	+++
Sclerotiniumoryzae	MTCC-3823	+++
Fusariumgraminearum	MTCC-5082	++
Rhizoctoniasolani	MTCC-5177	+
Aspergillusphonesis	MTCC-4012	+
Candida albicans	MTCC-4323	++

* +, inhibition zone 5-10mm; ++, inhibition zone 10 – 15mm; +++, inhibition zone> 15mm.

Figure1: IR Spectrum of purified antifungal fraction from Sephadex G75 column.