A tyrosinase inhibitor from *Aspergillus niger*

K. Y. Vasantha · C. S. Muruges · A. P. Sattur

Revised: 24 April 2014 / Accepted: 24 April 2014 / Published online: 14 May 2014
© Association of Food Scientists & Technologists (India) 2014

Abstract Tyrosinase, in the presence of oxygen, is the main culprit in post harvest browning of food products, resulting in the drop in its commercial value. In an effort to seek natural tyrosinase inhibitors for food applications, a screening programme was undertaken. Of the 26 fungal cultures isolated from soil samples of Agumbe forest, India, one isolate S16, identified as *Aspergillus niger*, gave an inhibition of 84 % against the enzyme. The inhibitor was isolated by following an enzyme inhibition assay guided purification protocol. The structure of the inhibitor was elucidated and found to be kojic acid. The IC₅₀ of the Competitive inhibitor was found to be 8.8 µg with a K_i of 0.085 mM.

Keywords Tyrosinase inhibitor · Screening · Kojic acid · Fungi · Food applications

Introduction

The enzymatic browning of plant-derived foods and beverages takes place in the presence of oxygen when tyrosinase and their polyphenolic substrates are mixed when cell structures are ruptured, during the brushing, peeling and crushing operations. These transformations destroy essential amino acids, lower nutritional quality and impair digestibility. A number of compounds capable of inhibiting enzymatic browning in food products through the interference of enzyme mediated reactions or through the reduction of *o*-quinones to *o*-diphenols have been reported (Schallreuter et al. 2008; Khan 2012). However, the repertoire of such chemicals that can be used in food systems is limited due to off-flavors, off-

odors, toxicity and economic feasibility. For example, in the past, sulfiting agents were widely used to prevent enzymatic browning in agricultural and seafood products. (Rescigno et al. 2002; Parvez et al. 2007). Due to health concerns of such agents, now most of the alternatives are formulations of ascorbic and citric acids, though they are less effective than sulfiting agents. since ascorbic acid is quickly consumed in the process of reducing quinones formed by tyrosinase. Another popular agent is 4-hexylresorcinol which is effective in prevention of shrimp melanosis and for browning control in fresh and dried fruit slices and is considered to be safe for use in the food industry (Radha et al. 1991; Adelmo et al. 2006).

As safety is of primary concern, there is a constant search for better inhibitors for use in the food industry. While many chemically synthesized compounds could be potent inhibitors, searching for inhibitors from natural sources is an attractive proposition as they are largely free of any harmful side effects. Hence, it is against this background that a screening programme for tyrosinase inhibitors from microbial sources was undertaken.

Materials and methods

Media and isolation of fungal culture

Sucrose broth, Sabroud's dextrose medium, Oat Meal Medium, Czapek- Dox broth, Glycerol broth, Yeast Malt Extract Broth, Malt Extract Broth and Nutrient Broth were prepared as described in the Handbook of Microbiological media (Atlas 1993).

1 gram of air dried soil samples previously obtained from Agumbe forest, Western ghats, India, were serially diluted and spread plated on the potato dextrose agar medium supplemented with sodium deoxycholate, 30 mg/L and chloramphenicol, 300 mg/L. Upon growth, the colonies were further

K. Y. Vasantha · C. S. Muruges · A. P. Sattur (✉)
Fermentation Technology and Bio engineering Department, Central
Food Technological Research Institute, Mysore 500020, India
e-mail: asattur@cftri.res.in

Table 1 The isolated cultures with their inhibitory activity against tyrosinase

S No	Culture number	% inhibition	S No	Culture Number	% inhibition
1	S1	Nil	14	S14	Nil
2	S2	Nil	15	S15	Nil
3	S3	9	16	S16	80
4	S4	27	17	S17	Nil
5	S5	Nil	18	S18	37
6	S6	Nil	19	S19	24
7	S7	25	20	S20	Nil
8	S8	4.7	21	S21	40
9	S9	Nil	22	S22	Nil
10	S10	19	23	S23	Nil
11	S11	Nil	24	S24	Nil
12	S12	Nil	25	S25	Nil
13	S13	Nil	26	S26	Nil

isolated on to freshly prepared PDA plates and finally transferred to slants.

Fermentation and Extraction of the inhibitor

A loopful of culture was used to inoculate on different media like Potato dextrose broth, Czapek Dox Broth, Sabaurod's Broth, Glycerol Broth, Sucrose Broth, Yeast Extract Malt Broth, Nutrient Broth, Oat Meal Broth, Malt Extract Broth (Himedia, India), previously sterilized at 121 °C for 15 min at 15 lb, and kept on rotary shaker incubator operating at 200 rpm at 28 °C for 7 days. At the end of fermentation, the broth was extracted with ethyl acetate (1:1, v/v) and kept on rotary shaker at 200 rpm for 2 h at 28 °C. The whole broth was filtered through Whatman No.1 filter paper to separate

Table 2 The amount of crude and the corresponding percentage inhibition produced by S16 in various culture media

Media	Weight of crude (mg/L)	Inhibition, %
Potato dextrose broth	217	80
Czapek Dox Broth	37	32
Sabaurod's Broth	31	-
Glycerol Broth	54	45
Sucrose Broth	16	16
Yeast Extract Malt Broth	112	17
Nutrient Broth	27	11
Oat Meal Broth	63	33
Malt Extract Broth	44	-

biomass from the broth. The solvent in the filtrate broth was separated by separating funnel and removed under reduced pressure by evaporation. The resultant crude extract was used as a source of the inhibitor.

1 gram of the crude extract was added to silica gel-G (60–120 mesh) packed in hexane onto a 50×1 cm glass column. Elution was done at 1 ml/min with various proportions of hexane, chloroform, ethyl acetate and Methanol. Fractions were collected and assayed for tyrosinase inhibition.

Tyrosinase assay and structure elucidation

The enzyme assay was carried out according to the method of Duckworth and Coleman (1970) using purified mushroom tyrosinase with a specific activity of 3,300 units/mg protein. A typical enzyme assay comprised 10 µL of enzyme; 10 µL of crude extract (inhibitor) dissolved in Di Methyl Sulfoxide (DMSO) and made upto 1 ml by 100 mM phosphate buffer (pH 6.8). In the control assay, the same volume of DMSO was used instead of the inhibitor. The enzyme reaction was initiated by the addition of 35 µL substrate (6.3 mM L-3,4-

Fig. 1 IC₅₀ values of crude, fraction 5 and purified Inhibitor**Table 3** Column eluted fractions of crude S16 culture and their percentage inhibition against tyrosinase

Solvent system	Colour of fraction	Fraction weight (mg)	% of inhibition
100 % Ethyl acetate (F1)	Yellow	0.5	-
10 % methanol (F2)	Light Yellow	-	-
10 % Methanol (F3)	Light orange	1	21
20 % Methanol (F4)	Brown	79	61
20 % Methanol (F5)	Light brown	80	84
20 % Methanol (F6)	Very light yellow	0.3	-
30 % Methanol (F7)	Colorless	-	-
50 % Methanol (F8)	Light yellow	0.8	13
75 % Methanol (F9)	Light brown	2	-
100 % Methanol (F10)	Brown	0.7	15

Fig. 2 The HPLC chromatogram of the purified inhibitor

Dihydroxyphenylalanine (DOPA)) and the increase in absorbance at 475 nm was recorded at the end of 3 min. The relative activity was expressed as the percentage ratio of enzyme activity in the presence of inhibitor with enzyme activity in the absence of enzyme inhibitors at the end of 3 min of enzyme reaction.

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker, DRX-400 MHz instrument at 20 °C. About 10 mg of the solid sample dissolved in DMSO- d_6 was used for recording the spectra. Infrared (IR) spectra were obtained in potassium Bromide pellets with a Bio-Rad FTS-135 spectrophotometer (Bio-Rad, Richmond, CA, USA). Electro spray ionization mass spectra (ESIMS) data were taken on a waters –3,000 mass spectrometer.

Results and discussion

While our focus of research is on tyrosinase inhibitors towards food applications, the ability of the inhibitors in reducing melanin biosynthesis also makes them popular in cosmetic products for hyperpigmentation-related concerns, including the formation of freckles. (Mahmud 2007; Nico et al. 2009).

From the air dried soil samples, appearance of fungal colonies were noticed in plates of 10^{-3} and 10^{-4} dilution within the first 3 days. The colonies had various morphologies such as cottony growth and smooth or wrinkled with spores of different colors. About 26 colonies were isolated and their corresponding crude extracts were screened for tyrosinase inhibition (Table 1). Some culture crudes like S3, S8 gave negligible inhibition. Isolates S7, S10, S18, S19 and S21 gave less than 50 % inhibition against tyrosinase. The highest tyrosinase inhibition activity was shown by S16 crude. In order to confirm its inhibition, a dose dependent assay was carried out and it showed an IC_{50} of 12.8 μ g (Fig. 1). This proved the presence of inhibitor and S16 was taken up for further purification studies. The isolate was further identified as *Aspergillus niger*.

In our experience of working with microbial metabolites, we have observed that fermentation medium is an important criterion to obtain sufficient quantities of crude extract and thus good amounts of purified inhibitor. Once S16 was selected as the inhibitor producing culture, various standard fungal media were tried and the crude extracts were assayed for tyrosinase inhibition (Table 2). It was seen that yeast extract malt broth gave a high crude content but the inhibition was less than 20 %. All the other media presented both low crude and inhibition, except potato dextrose broth (PDB) which had the highest crude amount and inhibition of 217 mg/L and 80 %, respectively. Hence, PDB was used as a medium of choice for purification of the inhibitor.

Purification of the inhibitor was effected by column chromatography where the crude extract was eluted with ethylacetate and methanol and with combinations therein. Fractions were collected on the basis of colour and assayed for their inhibition against the enzyme (Table 3). It was seen that almost the entire inhibitor was eluted with 20 % methanol in ethylacetate with fraction F5, showing the highest inhibition of 84 %. Fractions F4 to F6 were pooled and the inhibition was confirmed through a dose dependent assay, which showed complete inhibition 20 μ g and the IC_{50} at 8.8 μ g (Fig. 1). The active pooled fractions were loaded onto Sephadex LH-60 gel filtration column. In this second stage of chromatography, the partially purified fractions were eluted with methanol to get a pure white crystalline powder with an IC_{50} at 7.4 μ g (Fig. 1). The purity of the inhibitor was confirmed as a single peak at a retention time of 13.95 min by HPLC using C18 column (4.6X250mm) with Phosphate

Fig. 3 Structure of kojic acid

Fig. 4 Dixon plot of purified inhibitor

buffer and methanol (99:1) as mobile phase at a flow rate of 0.9 ml/min at 269 nm (Fig. 2).

The structure of the compound was elucidated by performing NMR, Mass and IR spectroscopy and was identified as Kojic Acid (Fig. 3). The inhibitor from S16 was compared with standard kojic acid wherein both showed the same retention time in HPLC (13.9 min) and m/z (143.1) by HPLC and MS with slight difference in the IC_{50} this may be due to the slight impurities in isolated kojic acid. Further, the mode of inhibition seen from Dixon plot indicated the isolated kojic acid to be a competitive inhibitor with K_i of 0.085 mM (Fig. 4).

Conclusion

While this work resulted in isolation of kojic acid, a well known inhibitor, it brings to highlight the pitfalls in the discovery of ‘new’ molecules from natural sources. While the literature on tyrosinase inhibitors from plant kingdom is huge, specially those discovered in the last five years, are classified into five major classes, including polyphenols, benzaldehyde and benzoate derivatives, long-chain lipids and steroids and other natural inhibitors (Chang 2009), the ones reported from fungal or microbial sources are far and between and the most commonly isolated one being kojic acid. This observation was further confirmed when purification of the S18 and S21 crude extracts, which showed 37 % and 40 % inhibition, respectively, against tyrosinase (Table 1) also yielded kojic acid.

Acknowledgments The funding for this work from the 11th Five Year Plan Network Project on “Exploitation of India’s rich microbial diversity” is gratefully acknowledged.

References

- Adelmo MG, Gustavo V, Barbosa C, Ralph PC, Arthur JM, Radha I (2006) Control of Browning During Storage of Apple Slices Preserved by Combined Methods. 4-Hexylresorcinol as Anti-Browning Agent. *J. Food Sci* 58:797–800
- Atlas RM (1993) Handbook of Microbiological media. CRC press, Boca Raton
- Chang TS (2009) An Updated Review of Tyrosinase Inhibitors. *Int J Mol Sci* 10:2440–2475
- Duckworth HW, Coleman JE (1970) Physicochemical and kinetic properties of mushroom tyrosinase. *J Biol Chem* 245:1613–1625
- Khan TH (2012) Novel Tyrosinase Inhibitors from Natural Resources - Their Computational Studies. *Curr Med Chem* 19:2262–2272
- Mahmud THK (2007) Molecular design of tyrosinase inhibitors: A critical review of promising novel inhibitors from synthetic origins. *Pure Appl Chem* 79:2277–2295
- Nico S, Jana V, Stan P (2009) The Hunt for Natural Skin Whitening Agents. *Int J Mol Sci* 10:5326–5349
- Parvez S, Kang M, Chung HS, Bae H (2007) Naturally occurring tyrosinase inhibitors: mechanism and applications in skin health, cosmetics and agricultural industries. *Phytother Res* 21:805–816
- Radha I, Craig W, Bohmont RJM (1991) 4-Hexylresorcinol and prevention of shrimp blackspot: Residual analyses. *J Food Comp Anal* 4: 148–157
- Rescigno A, Sollai F, Pisu B, Rinaldi A, Sanjust E (2002) Tyrosinase inhibition :general and applied aspects. *J Enzyme Inhib Med Chem* 17:207–218
- Schallreuter KU, Kothari S, Chavan B, Spencer JD (2008) Regulation of melanogenesis-controversies and new concepts. *Exp Dermatol* 17: 395–404