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Extraction and Purification of melanin from various cells and tissues

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Abstract

Melanins are phenolic biopolymers synthesised by most of the living organism mainly for photoprotection or surviving in harsh conditions. Melanin is localised in different areas or complexed with different other biomolecules when observed from animals to microbes. This makes the melanin extraction procedure different in animal, plant and microbial tissues. Basically, the alkali-acid extraction is used in most protocols of which slight variations are there depending on the tissue used. This review will try to compile melanin extraction procedures from different cells and tissues ranging from animals to bacteria.

Key words: melanin, extraction, isolation, animal, plant, microbes

Melanin is a ubiquitous pigment distributed throughout most of the living organism. In some organism (animals) it is located in specialised organelles called melanosome, while in some other (plants) it is widely deposited in seed coat, while deposited in cell wall in organism like fungi and secreted extracellularly in bacteria. Mainly there are four types of melanin depending on the precursors from which it is derived. These include eumelanin (most abundant melanin in human beings), pheomelanin (seen widely in humans and bird feathers), neuromelanin (seen in human brain) and heterogenous allomelanins which include pyomelanin (bacteria), DHN-melanin (fungi), catechol melanin (plants) etc. Melanin is a pigment with wide variety of properties makes it a suitable candidate to be utilized for numerous applications. Apart from its UV protective ability, melanin is a good antioxidant, metal chelator, antimicrobial agent, anti-inflammatory substance, having anti-cancer potential and lot more. This makes the pigment to utilized from cosmetic formulations to bioelectronics effectively (Narayanan *et al*, 2020).

Extraction of melanin is a very complicated process due to its diversity and structural complexity. The melanin is commonly extracted by break opening the tissue and extracting the melanin using alkali. The extracted melanin is precipitated using acid and further washed using organic solvents to get purified melanin. Depending on the type of tissue and localization of melanin the extraction procedure may vary slightly. These processes use corrosive chemicals and these chemicals may disrupt the structure of the polymer, many methods have been used so far for obtaining the purified pigment (Ghadge *et al*, 2022) This review is an effort to compile various melanin extraction procedures from different cells and tissues of different organisms.

Extraction from animal tissues

From Human melanoma and normal tissue melanin is extracted as per Watts *et al*, 1981.Human tissue is taken, adipose tissue is removed. Tissue is further dried in a stream of nitrogen. The tissue (150mg) is then homogenised glass-Teflon homogeniser with 5 mL distilled water. Further the sample is centrifuges at 10000 x g for 20 min and resultant pellet were extracted first with chloroform-methanol (2;1) and then with ethanol-ether (3;1) to remove lipids. The

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resulting residue is dried in nitrogen and then treated with 5 mL of lysing solution containing 0.3% saponin in 0.9% NaCI solution to remove haemoproteins. Samples are then centrifuged at 10000 x g for 20 min and to the resulting pellet is washed with 7.0 ml 5 mM $MgCI_2$ in 0.15M NaCI. The sample is then treated with 0.5% sodium dodecylsulfate in0.05M Tris buffer (pH7.0) containing 2mg/ml Pronase-CB and incubate at 30^{o}C for 48 hours. The digest is then centrifuged 10000 x g for 30 min. The remaining residue containing melanin granules is treated with 6.0ml 0.9% NaCI solution to remove the soluble impurities. The pellet is then washed in distilled water and further dissolved in 0.1 NaOH to get pure melanin.

The melanosomes present in human hair is tightly bound to a protein matrix called melanoprotein. Mainly two methods namely, acid/base extraction and enzymatic extraction are commonly used to separate melanin from keratin fibers. In acid extraction, hairs are cut into 5 mmm in size and to this add 7.2 N HCl (40 times the weight of the hair) at 107◦C in an oil bath or mantle heater under stirring and under nitrogen or argon flow for 4 hours. After incubation, the suspension is filtered out and the resulting residual fraction is washed with deionised water followed by acetone to get purified melanin granules. In enzymatic method the procedure is slightly different, Hair pieces of 5 mm size is treated with 60 mg/mL papain in 100mM phosphate buffer (pH 6.8) and incubate at 50^{o}C for 72 hours. After treatment the resulting suspension is filtered and resulting solid material is washed 20 vol% of IPA followed by IPA-n- Hexane-Water (6:6:1) and then with deionised water to get pure melanin (Ghiani *et al*, 2008). Joshi and Adivarekar, 2019 proposed ultrasound assisted alkaline hydrolysis to extract melanin from human hair. Hair sample is soaked in sodium hydroxide of different concentration which is further exposed to ultrasonic radiation in different time intervals. The solution is then filtered through sintered glass filter. The resulting dark black filtrate is treated with trichloroacetic acid and ammonium sulfate to crude melanin pellet. The melanin is then treated with urea to remove protein impurities and further dialysed to get pure melanin. Similar protocol could be utilized to isolate melanin from feathers of birds.

In the human brain-stem, there is region with brown to black colour called substantia nigra pars compacta where neuromelanin granules are concentrated. The protocol for the isolation of neuromelanin differs from other types of melanins. Firstly, disaggregate human substantia nigra pars compacta. Total of 5 gm of the brain tissue is taken for the experiments. Keep one piece of the tissue above a nylon mesh and press the tissue through the mesh using plunger of 10 mL syringe. Transfer the resultant suspension to a 50 mL conical centrifuge tube and maintain it in ice. Overlay 7.5 mL of suspension on to an ice-cold discontinuous sucrose gradient. Centrifuge at 4000 x g, 4^{o}C for 45 minutes. Dark brown neuromelanin pellet will be visible after centrifugation. Discard the supernatant and add 500µL of isolation buffer to the pellet. Homogenize the pellet by passing through a 1 mL syringe with a 26-G needle. Layer the homogenate over 5 mL of 80% Percoll solution. Centrifuge at 4400 x g, 4^{o}C to pellet down the neuromelanin granules. Further wash the pellet with 500µL of wash buffer and the isolated neuromelanin granules are stored in liquid nitrogen (Tribl, 2008).

Melanin extraction from molluscs *Patinopecten yessoensis* shells is done by the following procedure. The shells were carefully cleaned in water and powdered. 20 g of shell powder is treated with 6M HCl at 95-100^{o}C for 2 hours in a hot reflux equipment. The precipitated crude melanin is filtered using a Buchner funnel. The melanin is further packed in a Soxhlet apparatus and extracted with petroleum ether to remove lipid content. Remaining purified melanin is dried in hot air oven at 80^{o}C (Xiujun *et al*, 2017).

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Melanin is extracted from silkie and comb of silkie fowl and ink sac of octopus and sepia by the following procedure. 20g of sample was homogenized in 200mL of KOH which is further sonicated. The extract is then treated with hot alkali (100^{o}C) for 5 hours under reflux in an atmosphere of nitrogen. After the treatment the dark brown filtrate is acidified up to pH 2. The resulting crude melanin precipitate is pelleted by centrifuging at 10000 rpm for 10 min. The melanin is further hydrolysed using 40 mL of 7 M HCl for 2 hours at 100^{o}C.The nonhydrolyzable melanin redissolved in 20 mL of 1M KOH and 8 mL and 0.8 mL of chloroform and 1-butanol is added respectively. The mixture was shaken 30 minutes under nitrogen and further centrifuged 6000 rpm for 10 min. The chloroform phase is discarded and resulting alkaline melanin solution is reprecipitated using HCl. The resulting precipitate is dried into powder form (Harki *et al*,1997, Lin and Chen, 2004)

Extraction from plant tissues

Extraction of melanin from fruits and seeds of *Nyctanthes arbor-tristis* is done as per Kannan and Ganjewala, 2009. 10 g of dried seed or flower powder is added to 75 mL 2 M sodium hydroxide (pH 10.5) and incubate for 24 hours. The mixture was further centrifuged 8000 rpm for 15 min. The supernatant was acidified to pH 2.5 using 2 M HCl and further incubated for 2 hours. The resulting precipitate is pelleted out using centrifugation at 4000 rpm for 15 minutes. The precipitate is then subjected to acid hydrolysis using 6M HCl at 100^{o}C for 2 hours. The precipitate is further treated subsequently with chloroform, ethyl acetate and ethanol following redissolving in 2M sodium hydroxide. The melanin is again reprecipitated using 1M HCl and further dried to get purified melanin.

Extraction from Fungi

Extraction of melanin from black yeast *Hortaea werneckii* is done as per the following protocol. The yeast is grown in potato dextrose agar plates. Disc of 15 mm diameter of 15-day old culture were cut and boiled for 5 min in 5 mL distilled water. Centrifuge the sample to remove the cell debris and added 3 mL of 1 M NaOH and autoclave (120^{o}C for 20 min). The resulting pigment solution is acidified using HCl and the resulting precipitate is washed 2-3 times with distilled water to get pure melanin (Rani *et al*, 2013).

Isolation of melanin from the conidia of *Aspergillus fumigatus* follow the following procedure. Conidia of 5-day old culture from SDA plates is collected. The conidia is washed in PBS three times and further washed in 1 M Sorbitol and 0.1M sodium citrate (pH 5.5). 10 mg/mL of Novozyme (cell wall degrading enzyme) is added and incubated at 30^{o}C overnight to generate protoplasts. The protoplasts were collected by centrifugation and further washed with PBS and treated with 4 M guanidine thiocyanate overnight. Resulting dark particles are washed with PBS and treated with 1 mg/mL Proteinase K in reaction buffer containing 10mM Tris, 1mM $CaCl_2$ and 0.5%SDS, pH7.8. The resultant debris was the washed 3 times with PBS and boiled with 6 M HCl for 1-5 hours. Melanin particles were then filtered through Whatman and washed extensively using distilled water. Particles were dialysed against distilled water for 10 days until acid residue is removed and further lyophilized to the purified pigment (Youngchim et al, 2004).

Extraction from bacteria

Compared to all other cells and tissues isolation of melanin from bacteria is the easier procedure. Bacteria secrete melanin extracellularly which makes it easier to be precipitated

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from the culture media. After the melanin production the bacterial cells are firstly pelleted out by centrifugation at 5000 x g for 10 minutes. The cell free supernatant obtained will be used for further extraction. The cell free supernatant is acidified using 1N HCl up to pH 2 so that precipitated melanin could be visible at the bottom of the flask. The precipitate is allowed to stand for a week under sterile conditions at room temperature for complete precipitation, followed by boiling it for 1 hr and further centrifugation at 5000 x g for 10 minutes. Resulting pellet is washed with 0.1 N HCl followed by distilled water. To the melanin pellet add 10 mL of ethanol and keep it in boiling water bath for 10 minutes. Then keep it for 1 day and further wash twice with ethanol and then air died (Sajjan *et al*, 2013; Kurian & Bhat, 2018).

Due to corrosive nature of acids and alkali used in the procedures for melanin extraction the process become economically less feasible. Ghadge *et al*, 2022 recently came with a efficient technique which use organic solvents to extract actinomycetes melanin without using acid. The protocol is as follows; 8 day grown melanin produced culture medium is centrifuged to remove the microbial biomass. The supernatant is further free dried and this powder is used for further melanin extraction. 20 mg of lyophilised powder is mixed with 1 mL distilled water followed by the addition of 1 mL of tetrabutylammonium hydroxide (40% w/w TBAOH in water) and stir it for 30 min. To this mixture add 1 mL ethyl alcohol and allow it to stand for 1 hour and further centrifuge at 13000rpm for 10 min. resulting pellet is washed with ethyl alcohol twice and the pellet is lyophilized to get the purified melanin.

In conclusion, common procedures with slight changes according the tissue types have been used to extract melanin from different cells and tissues. Most of these procedures use costly enzymes and corrosive chemicals which could be the main drawback in the downstream processing of melanin. To make the purified melanin economically feasible more techniques like TBAOH extraction need to be explored. This makes melanin extraction still a less explored area in pigment research.

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