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REVIEW ARTICLE

Biochemical and Molecular Characterization of Microbial Keratinase and Its Remarkable Applications

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ABSTRACT

Microbial keratinase have become biotechnologically important enzyme since they target the hydrolysis of highly rigid, strongly cross-linked structural polypeptides "Keratin" recalcitrant. Keratins are insoluble fibrous proteins found in hair, wool, feather, nail, horns and other epithelial coursing contains beta helical coil which is linked through cysteins bridges. Keratinases, which are produced by several bacteria, fungi and actinomycetes that have been often isolated from terrestrial and marine sources. The bioconversion of insoluble feather keratin to soluble feather residue has high nutritional values and can be employed as a supplement for livestock feeds. Other promising applications have been associated with keratinolytic enzymes, including elimination of keratin in acne, depilation process, preparation of vaccinine for dermatophytosis therapy, pharmaceutical enhancement of the nail treatment and degradation of prion and prion - like proteins.

Key words: Keratin, microbial keratinase, recalcitrant, applications.

INTRODUCTION

Keratinase [E.C.3.4.21/24/99.11] are proteases able to degrade the scleroprotein keratin. Huge amount of this protein are available as feathers, a waste by product generated by the poultry production [1]. Keratinases are very promising in a biotechnological point of view, since they could be applied to process keratin containing waste from feather and poultry industries and to improve nutritional value of feather meal. Insoluble and hard to degrade animal proteins are ubiquitously present throughout animal bodies. E.g. hails, horns, hair, wool and feather. Feather wastes are generated in large funatities as a by product of commercial product. Feathers represent 5-7 per cent of the total weight mature chickens and its made up primarily of keratin. It is resistant to common proteolytic enzymes and poorly digested by most organisms. However, it does not accumulate in nature [2]. The mechanical stability of keratin and its resistance to biochemical degradation depends on tight packing of the production chains. In α – Helix (α-keratin), or β – Sheet (β – keratin) structure are linked by disulfide bonds which fold into initial 3-dimensional form. The enzymes capable of degrading keratin are known as keratinolytic enzymes [3].

Keratin utilization has been reported in variety of organisms including non-filamentous and filamentous bacteria, water moulds and filamentous fungi. Along with bacteria and fungi, some insects including cloth mouth leaves, carpet beetles are known to digest keratin [4]. The complex mechanisms of keratinolysis involve cooperative actions of sulfitolytic and proteolytic system. It is robust enzymes with a wide temperature and pH activity and is largely serine or metallo proteases. Sequence homologies of keratinases indicate their relatedness to subtilites family of serine proteases. It could reduce the high degree of cross-linking in keratinous proteins easily occur on the wool surface, thus it was considered as a potential enzyme for wool modification [5].

Recombinant Keratinase

A recombinant strain, *Bacillus subtilis* FDB-29, which carries the P43 promotor and over express Ker A was subsequently developed by genetic transformation [8]. Since the first report on the alkaline protease from alkaliphilic *Bacillus clausii* 221 and that there have been extensive studies on alkaline proteases from alkaliphilic *Bacillus* such as Yab NKS-21 [6]. This enzyme was found to be most active towards casein at pH 12-13 and was

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stable for 10 min at 60ºC in the pH range 5-12. This enzyme readily digests human hair and nail in alkaline buffer (pH 11-13) containing one per cent triglycolic acid [7] and offers great advantage for industrial processes such as leather, tanning and wastewater treatment and for domestic production such as depilation. The alkaliphilic *Bacillus sp* AH-101 is identified as *B.halodurans* C-125 by genome sequencing that will be useful in developing further industrial applications, particularly its extremely thermostable alkaline keratinase [9]. The expression of genes from thermophilic bacteria is not always enhanced at elevated temperatures. Poor stability of the mRNA of keratinase at higher temperature was observed. The ker A expression system may be heat sensitive. From equal amount of total RNA, mRNA of Ker A was much lower in *B. licheniformis* cells at 50ºC than at 37ºC in flask cultures, at 37ºC *B.subtilis* over expressed keratinase by 3 to 4 fold compared with *B.licheniformis* at 50ºC [10].

Several reports discussed the isolation, purification and characterization of keratinolytic enzymes from bacteria, actimoycetes and to lesser extent from fungi [11]. Few attempts have been made to clone keratinase genes from keratinolytic microorganisms. Low expressions as well as low stability of the recombinant plasmids carrying keratinase genes are considered obstacles to maximize the amount of keratinase produced by genetically modified cells [12]. High expression level of a keratinolytic serine alkaline protease (apr E gene) from the *B. subtilis* DB 100 (pH 5.2) recombinant strain was reported [13].

The mutant stain KD-N2 producing keratinolytic activity about 2.5 times that of the wild-type strain. It produced inducible keratinase in different substrates of feathers, hair wool and silk under submerged cultivation [14].

Biochemical and molecular characterization of keratinase

The properties of microbial keratinases may diverse depending on the producer microorganism. These enzymes are predominantly extracellular [15]. Mostly keratinases belong to the subtilisin family of serine with cysteine protease, which have higher acvivity on casein [16]. Metal ion may be acts as a salt or as an ion bridge to maintain the confirmation of the enzyme substrate [17]. Some general biochemical characteristics of selected keratinase are presented in (**Table 1**).

Table 1: Biochemical characteristics of selected keratinase

Microorganisms	Catalytic type	Mol.mass (KDa)	Optimal pH	Optimal Temperature (°C)	References
Bacillus halodurans PPKS-2					
Keratinase - 1	Disulphide reductase	30	11.0	60-70	[18]
Keratinase – 2	Serine protease	66	11.0	70	
Bacillus sp	Serine	32	8.0	50	[19]
*Bacillus sp.*JB 99	Serine protease	66	10.0	65	[20]
Pseudomonas sp.,MS 21	Serine	30	8.0	37	[21]
Pseudomonas aeruginosa	Serine	33	7.0	50	[22]
*Vibrio sp.*Kr 2	Serine	30	8.0	55	[23]
Doratomyces microspores	-	45-70	9	-	[24]
Streptomyces sclerotialus	-	46	9.0	55	[25]
Streptomyces strain BA 7	Serine	44	8.5	50	[26]
Aspergillus oryzae NRRL-447	-	39.7	7	70	[27]
Penicillium sp	Metallo	19	6-8	50	[28]
Ahm 1	Protease	40	6-11	60-65	
Ahm 2					
Chrysobacterium sp.strain Kr 6	-	20	-	50-60	[29]

Most of the microbial keratinases are alkaline or neutral proteases showing optimum pH ranging 7.5 - 9.0 and 40 - 85ºC. This property is remark for the keratinase of *Nocardiopsis* TOA-1 which is stable over a pH range of 1.5 to > 12.0 for 24 h at 30ºC. *S. albidoflaris* produces a chymotrypsin like keratinases that exhibited specificity for aromatic and hydrophobic amino acid residues, as demonstrated by using synthetic peptides [30].

The catalytic type of many keratinases has been determined by using specific substrates and inhibitors [31]. Cell and colony morphology, growth characteristics of the isolate were also coincident with the properties of *Stenotrophomonase maltophilia* DHHJ. Alkaline protease from strain *Nocardiopsis sp* TOA 1 designated NAPase was between 70 - 75ºC and optimal pH was 11.0 - 11.5. NAPase was found to be stable below 60ºC after 10 min of incubation. The molecular mass of NAPase estimated as 20kDa and the isoelectric point was above pH 10.0 [32].

The high activity of *Trichophyton mentagrophlesvar* keratinase on guinea pig hair

and fibrous protein can be related to its substrate specificity or due to the removal of some accessory production that are capable of splitling the di-sulphide bonds presents in keratinzed production during the purification process. The keratinase produced by *S.sclerotialus* showed strong inhibitory effect against metals like $MgS0_4$, $Cacl_2$. The optimum temperature and pH recorded 55ºC and 9.0 respectively. The enzyme was strongly active on L-Leu (amino - 4 - methycocmain) (Amc) and N-t-moc-Ile-Glu-Gly-Arg-AMC, showing preference for hydrophoblic and positive amino acids kinetics of thermal inactivation of *Chyseobacterium* kr6 keratinase was determined by incubating the enzyme with different purification grades. The termal denaturation of enzyme is accompanied by weakening or disruption of non-covalent linkages either simulateous increase in the enthalphy of activation or entrophy [33].

In summary the multiplicity of catalytic mechanisms observed for microbial keratinases (serine, thiol and metalloproteases) could have an important effect in the natural environment. Keratinase adsorption to fibrous keratin was previously showed to occur through electrostatic interactions [34].

The molecular weight determination and homogeneity test were carried out by SDS-PAGE using 10% polyacrylamide gel and stained with coomassie brilliant blue R 250. Zymogram was carried out. The sequence was analyzed by DNASTAR. The BLAST algorithm was used to search for homologous sequence in genbank. Molecular plylogenetic studies of the 16S rRNA were conducted using MEGA at the end [35].

Keratin Structure

Keratins are fibrous proteins which compose the structures and large portions of the cell compositions of living organisms [36]. There are two primary groups of keratins, the alpha-keratins and the beta-keratins. While both fulfill similar roles, they differ slightly in structure, composition and properties. The alpha-keratins are slightly basic or neutral and a right handed helical structure and the beta keratins are slightly acidic and also form a right handed helical structure. Keratins are composed of aminoacids, primarily the amino acid glycine and alanine.

Cross linkage

Cross linkage occurs with all the keratins. The cross-linkage occurs with hydrogen bonds formed between O_2 and H_2. It is the cross-linkage that allows layers upon layers of these chains binds together in sheets which gives structure. The cross-linking of keratins may play a role in epidermal differentiation. Since keratins from more differentiated areas of epidermis may contain a higher level of interchain isopeptide bonds. Keratin may be cross-linked itself or to stratum corneum form both soluble and insoluble aggregates. No adducts between keratin and purified cornified envelope were formed when both were treated with active epidermal transglutaminase [37].

Human Keratin

In human fifty four functional keratin genes exits. Keratins from obligate heteropolymers (between one type I and II keratin) and share a common structure consisting of a central coiled α helical rod domain that is flanked by non-helical head and tail domains. The α helical rod domain is subdivided into four sub domains (Coils 1A, IB, IIA and IIB), which are connected with three linkers (L1, L2 and L2). Most post-translational modified sites are found with in the head and tail domains. Keratins have long been recognized as diagnostic markers in tumor pathology [38].

Molecular weight of human keratin

The molecular weight of keratins in mammals ranges from 40 to 70 kDa was determined by two-dimensional gel electrophoresis and immunoblotting and compared with human bovine keratins and found that some keratins in corresponding tissues had the same molecular weight, others did not. For example, the suprabasal keratin K10 has the same molecular weight (56.5 kDa) in both human and bovine epidermis, whereas K13 has a molecular weight of 51 kDa in humans and 43 kDa in bovines. Our interpretation of this apparent is that keratins in corresponding cells and tissues of different mammalian species may have similar functions within the cells as well as antigenetic epitopes that bind the same antibody but that they actually differ in molecular structure and amino acid composition [39].

Sources of Keratin

Keratin is a fibrous structural protein found in human skin, hair and nails, it is also a part of the animal kingdom and found in birds, reptiles, amphibians and mammals. It is used in hair care products, animal feed and fibres for textiles (**Table 2**) [40].

Table 2: Sources of keratin

Sources	Uses
Animal Hooves	Glue and Hair care production.
Powdered Hooves	Unrestricted agent in Animal feed.
Sheep Hair	Human tissue repair, Biomedical uses, Personal care product.
Spider web Silk (β-Keratin)	Sutures for eye surgery, Artificial tendon and ligaments for knee surgery.
Human Keratin	Foods, Clothing, Skin care production, dirt production Diagnostic marker in tumour.

Source of keratinase

Upto now, a limited number of studies have been reported on the isolation of thermophiles, in particular thermophilic actinomycetes with the ability to hydrolyse wool and other keratinous wastes. Thermophilic actinomycetes have some advantage in comparison with mesophilic strains, such as accelerated accumulation of biomass and enzymes [41]. It has the ability to breakdown many different varities of organic production and is crucial in the mineralization of organic matter. A number of microorganisms have been reported could degrade different source of keratin, mainly bacterial actinomycetes, saprophytic and dermatologist fungi have been reported to exhibit keratinolytic properties. Kertinase from many bacrteria have been isolated and characterized. Some species of dermatophytes, including *Trichophyton mentagrophytes, T.rubrum, T.gallinae, Microsporum canis and M.gypseum* [42].

Keratinolytic enzymes that may have potential roles in biotechnological processes that involoving keratin containing wastes from poultry and leather industries. Dymatic hydrolysis by micro organisms possess keratinolytic activity represents an attractive alternative to improve the nutritional value of feather waster. The potential use of keratinase is in different application where keratins should be hydrolysed such as the leather and degtergent industries, textiles waste bioconversion, medicine and cosmetics for drug delivery through nails and degradation of Keratinzed skin, besides it involves in the hydrolysis of prion proteins that arise as novel outstanding applications of the enzyme [43].

APPLICATIONS

Degradation of Prion and Prion- like proteins

The degradation of prion and prion like proteins by microbial keratinase was reported. Prion diseases are a group of progressive condition that effect the brain and nervous system. Misshaped prion production carries the disease between individuals and cause deterioration of the brain. In recent times a type of TSE Transmissible Spongiform Incephalopathies (TSEs) called bovine spongiform encephalopathy (BSE) spread in cattle. It can not transmitted through the air or through touching or casual contact. It is transmitted through contact with infected tissue, body fluids or contaminated medical instruments [44]. Bacterial keratinase produced by *B. licheniformis* strain PWD 1 degreade prion protein (PrP) in brain, stem tissue from animals with bovine spongiform encephalopathy and scrapie degradation of surrogative yeast prion protein. Keratinase from *Nocardiopsis sp* TOA-1 effectively degraded a scrap prion production without chemical or physicl treatments prion proteins (PrP) are causative agents of transmissible spongiform encephalopathy (TSE), a fatal and transmissible neurodegenerative disease. BSE in cow, scrapie in sheep and goat, chronic wasting disease (CWD) in deer; transmissible mink encephalopathy (TMC) in mink and kuru and creutz feldt Jacob diease (CTD) in human are types of TSE. Three anaerobic thermophiles belonging to the *Thermoanaerobacter, Thermosipho* and *Thermococcus* subspecies were found to hydrolyze efficiently the thermally denatured amyloid recombinant prion and were selected as candidates for decontaminating amyloid aggreagates in animal wastes. Moreover, the *thermoanaerobacter subspecies* S 290 was shown to hydrolyze the prpsc deposits in mouse brain without denaturing treatment. Further research is to be conducted based on the results from the bioassay to optimize the reaction conditions necessary for the relevant enzymatic degradation of Prion Production [45].

Depliation process

Keratinous wastes could be degraded by some microorgnisms in nature. Native human foot skin (NHFS) was used as sole nitrogen source to screen microorganisms with keratin degradating capability [46]. For appromimately 200 strains, a strain of *Streptomyces sp* strain No 16 was found to possess the strongest keratinolytic activity and the substrate specificity test indicated that the crude keratinase could degrade keratin azure, human hair, cock feathers and collagen. This is the first report on specific induction of keratinase by NHFS from actinomycetes. Three novel keratinolytic *Bacillus Sp* isolated from the amazon basin have been recently characterized. These bacteria shared elevated homology with *B.Subtilis, B.amyloliquefaciens* and *B.velesensis* and produced a mixture of proteolytic activity that

produced a mixture of proteolytic activites that showed remarkable dehairing activity of bovine pelts. A combined treatment method of cutinase, keratinase and protease was applied in the wool processing to modify the wool properties. This combined action of this enzyme on wool obviously improve the wetability and anti felting property of wool fabrics. Keratinase could reduce the high degree of cross linking in keratinous proteins and make the proteolysis with protease easily occurs on the wool surface [47].

B.cereus Fk 26 was the least feather degrading strain degrading 99 per cent feather at 37ºC, 200 rpm. Keratinase materials such as feather wool and hair are insoluble resistant of degradation by common proteolytic enzymes. Feathers represent over 90% protein, the major component proteins extensively cross-linked by disulfide, hydrogen and hydrophobic bonds. Thus the several million tons of feathers generated annually by the livestock industry leads to some environmental pollution and wastage of protein-rich reserve. A keratinase, produced biotechnologically by *Doratomyces microsporus*, was used to treat porcine skin invitro under different experimental conditions [48]. Keratinase hydrolysed the outer epithelial sheath of hair roots provoking depilation. These data suggests the potential of this enzyme for application in eco-friendly leather processing [49].

Pharmaceutical enhancement of the Nail treatment

The tropical therapy of Nail disease is limited by the low permeability of drugs through the nail plate. To increase drug penetration, the integrity of the nail plate must be compromised to a certain extent. Keratinolytic enzymes might decrease the barrier properties of the nail plate by hydrolyzing the nail keratins. Then the addition of a reducing agent (Dithiothreitol, DTT) would increase the damage caused to the nail plate as it cleaves the disulphide linkages of keratins and increases access of the enzyme substrate. So the enzymatic disruption of nail plate is translated into enhanced drug penetration, into the nail plate [50]. Permeation studies using modified Franz diffusion cells and bovine hoof membranes as a model for the nail plate showed that the enzymes enhanced drug permeation through the hoof membrane. The permeability and partition co-efficient and the drug reflux were found to be significantly increased in the presence of the enzyme. From this hypothesis, the hydrolytic action of keratinase on nail plate proteins could increase ungula drug delivery [51].

Dermatophytosis therapy

Dermatophytosis or ringworm is a clinical condition caused by fungal infection of the skin in humans, pets such as cats, and domesticated animals such as sheep and cattle. The fungi that cause parasitic infection (dermatophytes) feed on keratin, which is found in the outer layer of skin, hair and nails. *Microsporium canis* is the main causative agent of dermatophytosis in animals. The use of extracellular enzymes to penetrate through the solid structural barriers in the host for the treatment against fungal pathogens [52]. Dermatophytes commonly gain entry into the host via (keratin rich) hair, skin or nails. Thus a great deal of attention has been focused on keratinolytic protease (keratinase) as a possible target for treatment of dermatophytosis. On the mechanisms of action of griseofulvin in dermatophytosis, guinea pig hair absorbed or adsorbed with griseofulvin was less efficiently hydrolyzed by the extracelluar and cell bound keratinase of *Trichophyton mentagrophytes* than untreated control hair. The same observations were made using hair from guinea pigs treated orally with griseofulivn .The antibiotic and three related compounds had no effect on the active sites of the keratinases. The results revelated to dermatophic attack is the result of griseofulvin to incorporation into keratinous structure casuing substrate modifications (*Microsporum sp AP1 Epidermophyton Sp-AP2, Trichophyton rubrum AP4, Trichophyton mentagrophytes AP5 and a yeast candida albicans AP3*). Among all these dermatophytes, *C.albicans* AP3 surived at 80ºC and exhibited great potential to elaborate keratinolytic enzymes. This high thermostablility of the enzymes are important attributes in the consideration of preventive and therapeutic methods against dermatophytes in the tropics [53].

Elimination of Keratin in Acne

The primary defect in acne is thought to be that there is too much keratin produced at the exist of the hair shaft. This blocks the surface and does not allow the sebum out. So the development of acne occurs. Keratinase proceed from *Bacillus licheniformis* (PWD) and *E.Coli* has been adapted to the laboratory and cosmetic applications especially acne treatment [54]. The keratinase (Ker A) from *B.licheniformis* (PWD) is active its molecular weight and isoelectric point is found to be 38.9 and 8.73 KDa [55, 56]. Keratinase is included in facial scrubs, and it is useful for treating acne, because dead cells can clog pores and create a favorable environment for acne. Some anti-dandruff shampoos include keratinase,

because the keratinolytic action allows shampoo to wash away flaky dead cells. It can eliminate warts, calluses and corns. The U.S food and drug administration (FDA) has approved keratinase for treating psoriasis, a condition involving excessive turn over of skin cells and scaly build up [57, 58].

CONCLUSION

Keratinases are proteolytic enzyme which is produced only in the presence of keratin. Keratinase produced by fungi, bacteria and actinomycetes are alkaline and thermophilic in nature. The keratinase could be applied for the detergent industry, dehairing for leather industry, cosmetic and medical industry. Application of human keratin markers in the cancer treatment are currently under investigation.

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