# Physiology of keratinophilic fungi

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*Summary* Most important pieces of knowledge on physiology related to keratinolytic ability of dermatophytes and related keratinophilic fungi are summarized. Research into carbon, nitrogen and sulphur metabolism, protease, lipase and esterase production is reviewed.

*Key words* Keratinophilic fungi, Physiology, Dermatophytes

The physiology of keratinophilic fungi has received relatively little attention, particularly over the last two decades. Relevant results are divided among a great many papers, often dealing primarily with other topics. Moreover, the vast majority of studies were concerned only with the keratinophilic fungi which are important for medicine, viz. the dermatophytes.

In this review I will summarize the most important information in a generalized form but will not describe the original data. Due to limited space, the scope of the paper is restricted to those aspects of physiology which are related to keratinolytic ability, which is the most important characteristic of keratinophilic fungi. For this reason, some traditional aspects of physiology, e.g. the use of sugars, the effect of growth factors, the production of antibiotics and aspects of pathogenicity in dermatophytes had to be omitted.

The term "keratinophilic fungi" is used for all fungi for which keratinized substrates are the natural habitat and "keratinolytic fungi" for fungi in which the degradation of native hard keratin has been experimentally proven. The letters T, M and E are abbreviations for the generic names of dermatophytes *Trichophyton, Microsporum and Epidermophyton*.

## Carbon metabolism

Keratinophilic fungi are adapted to the utilization of proteins as the main or sole source of nutrition. These fungi tend to utilize amino acids, peptides and proteins as carbon sources even in the presence of sugars (see section entitled 'Sources and metabolism of nitrogen'). In contrast, their ability to utilize polysaccharides and lipids is somewhat reduced.

Philpot [1] was unable to demonstrate the hydrolysis of starch by means of plate tests on 22 species of dermatophytes; dextrin was just a bad carbon source. Calvo *et al.* [2] found amylase in only 3 out of 23 strains of *T. mentagrophytes*. However, other authors have described starch utilization and amylase production [3-6]. Ziegler & Böhme [4] concluded that amylolytic ability was in dermatophytes, in comparison to the common soil fungi, rather rudimentary and typical were great differences among various species and strains. Polyfructosan inulin was not utilized as carbon source either in dermatophytes [4] or *Scopulariopsis brevicaulis* [7]. Native cellulose and its soluble derivatives and products like cellophane cannot be hydrolyzed by keratinophilic fungi, and the same is true for pectins [4]. As for the utilization of simple sugars, the studies [1] revealed no special feature to distinguish keratinophiles from other groups of fungi.

Because keratinized tissues can be relatively rich in lipids, some authors have studied the utilization of lipids as nutrients. Böhme [8] reported the ability of *M. gypseum* to grow on 10 emulgated lipids in the presence of glutamic acid as a source of nitrogen. Growth was best on oleum pedum tauri and wool fat followed by Tween 60 and Tween 20. Lipids extracted from "vernix caseosa" on the skin of neonates could also be used [9]. Hellgren and Vincent [10] found olive oil to be the best lipid C source for four species of dermatophytes. Tween 60 (polyoxyethylenesorbitan monostearate) was hydrolyzed better than Tween 80 (oleate). Tween 40 (palmitate) and Tween 20 (laurate) were very poor C sources and tributyrin was never utilized. The utilization of some other lipids was also studied [11-14]. In summary, dermatophytes can use common lipids as sources of carbon, but their lipolytic activity is only moderate to weak. It must also be remembered that the hydrolysis of lipids releases free fatty acids, many of which inhibit the growth of dermatophytes [15].

## Sources and metabolism of nitrogen

Keratinophilic fungi are rather exceptional among fungi in that they can use proteins as a sole source of carbon and nitrogen. Proteinaceous substrates contain a relative excess of nitrogen over carbon. They have up to 16% N, whereas in dry weight of dermatophyte mycelia it is only 5% to 6% [16]. In a similar way to other protein-degrading microorganisms, keratinophilic fungi get rid of excess nitrogen by intensive deamination and ammonia production. This causes the well-known tendency of keratinophilic fungi to alkalinize when they grow on media that contain proteins, peptides and/or amino acids. Denaturation by alkali represents a part of the biochemical

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mechanism of keratinolysis (see section entitled 'Keratin degradation'). In cultures on media with various proteins, peptides and amino acids, the pH of the cultivation fluid is commonly around 8 [16-21]; in cultures on keratin it may exceed 9 (see below). Any further increases in pH are limited by the volatility of ammonia, which escapes from alkaline solutions in gaseous form.

Deamination in the mycelium is performed by L-amino acid oxidases. These enzymes convert amino acids into keto (oxo) acids and ammonia. Oxygen consumption in the deamination process obviously makes the keratin degradation by fungi depend very much on aeration. As reported by Refai and Rieth [22], dermatophyte strains growing submersedly in liquid cultures alkalinized the medium only slightly. L-amino acid oxidases were demonstrated in the mycelium of dermatophytes [23] but they were not studied in detail. The deamination of cleavage products of proteins also takes place extracellularly in the medium, as shown by the fact that the production of ammonia and the rise in pH continue in the cultivation fluid after the mycelium has been removed. Actively secreted enzymes or enzymes released on autolysis of older hyphae may be involved. Although keratinolytic fungi often create an alkaline milieu around their mycelia, their pH optimum is near-neutral. In cultures on liquid media the growth of dermatophytes was optimum in pH 6.5 to 7 [16,24-26] and the same is probably true for all keratinophilic fungi. These fungi are therefore rather more alkalitolerant than alkaliphilic. In media which are rich in sugars they can even acidify the medium up to pH 4. However, this inhibits further growth [21,27]. In buffered media the dermatophytes grow well only in pH 6 to 7.5 but in nonbuffered media they can start growing in a broad range of pH 4 to 11 [19,25,28].

The dermatophytes are adapted to the use of proteins, peptides and amino acids as the sources of carbon. The above compounds are utilized preferentially from the medium even in the presence of glucose [16,19,24,29,30]. This results in a transient excess of nitrogen leading to deamination, production of ammonia, and a rise in the pH of the medium. As soon as peptides and/or amino acids are consumed, the fungus utilizes glucose together with $NH_4^+$ ions. Consequently the pH of the cultivation fluid decreases and its fall to below the initial value coincides with the end of the exponential growth phase. A renewed increase in pH is frequently observed in autolysis, and this is again accompanied by the production of ammonia.

Whereas keratinophilic fungi in nature are, by competing microorganisms, limited to the decomposition of keratinaceous substrates, in culture they can utilize other scleroproteins (e.g. collagen and elastin) and many soluble proteins. Proteins may serve as the sole sources of organic nutrition. Supplementation with glucose, however, always accelerates the growth and increases the maximum dry mass. The presence of sugars improves the C:N ratio and may also prevent over-alkalinization of the medium. Products of partial protein hydrolysis like peptone or casein hydrolyzate are very good sources of nutrition. They support excellent growth in the presence of glucose and may also be used as sole nutrients.

A great many authors have studied using amino acids as sources of nitrogen, mostly with glucose as the carbon source [7,25,31-42]. Individual amino acids were suitable not because of their abundance in keratin but of their position in the metabolic pathways. The best N sources were glutamic acid and glutamine and the members of urea cycle arginine, ornithine and citrulline. The utilization of asparagine, proline, serine, alanine, glycine, histidine and tyrosine was moderate and varied widely between species and strains. Amino acids with an extraordinary structure which were isolated in the metabolic pathways as tryptophan, valine, isoleucine and phenylalanine were poor nitrogen sources. The sulphur amino acids, methionine and cysteine, were not widely utilized. Good sources of nitrogen also served as the only sources of C and N, but growth was always better in the presence of well-utilizable sugars. Mixtures of 3 to 5 amino acids usually supported better growth than any compound used alone.

Urea can be utilized as a source of nitrogen and supports moderate to good growth [11,25,43-46]. Growth on media with urea and the increase in pH of the medium due to the production of ammonia is usually taken as proof of urea utilization and urease production. The results of this "urease test" vary considerably among species of dermatophytes, however. These differences are used in species diagnostics, particularly for distinguishing urease-positive *T. mentagrophytes* from the low producer of urease, *T. rubrum* [1,43-45].

Inorganic compounds have been known for a long time to be poor sources of nitrogen for dermatophytes. This holds fully true for nitrate and nitrite, which enable hardly any growth [1,11,25,35,42]. Accordingly, Czapek-Dox agar containing sodium nitrate is quite unsuitable for these fungi [2]. Ammonium salts can be utilized depending on the compound and concentration. With ammonium sulphate or nitrate the consumption of ammonium ions causes a strong acidification of the medium and inhibits growth. However, with ammonium phosphate or tartrate and/or buffering of the medium, the growth can be moderate to good [1,11,16,25,35,40,46,47]. It is not known whether the limited use of inorganic N sources is common to all keratinophilic fungi. In a keratinolytic strain of *Scopulariopsis brevicaulis* not only ammonium ions but also nitrate and nitrite were as good as glutamic acid as sources of nitrogen [7].

## Sources and metabolism of sulphur

The metabolism of sulphur is very important in keratinophilic fungi, because keratin is a sulphur-rich substrate and numerous disulphide bridges are the main source of its high resistance.

Stahl *et al.* [48] have already reported that cysteine, cystine, methionine and inorganic sulphate can serve as sources of sulphur for the growth of *M. gypseum*. Kunert [49] studied 10 inorganic sources of sulphur in the same dermatophyte. The best sources were sodium sulphate and sulphite, but pyrosulphite, persulphate and dithionite were also good. Growth on thiosulphate and tetrathionate was poorer and sulphamate did not serve as a source. Sodium sulphide (1mM) strongly inhibited growth, which only began after a long time lag.

Sulphur amino acids have been studied mainly just as sources of nitrogen, usually in glucose-containing media [35,38,48,50-55]. Methionine was a very bad source of nitrogen for the dermatophytes. Although in concentrations of up to 1 mM it can be a good source of sulphur, in higher concentrations it is strongly inhibitory [48,50,53,56]. Free cysteine is also a bad source of nitrogen and can inhibit growth when added to an otherwise suitable medium. This effect, observed in concentrations above ca. 2 mM, is probably due to the reactivity of the sulphydryl group and its capacity to reduce. In media supplemented with cysteine, morphogenetic effects and even growth in the form of yeast-like cells were described [51,57]. These effects were probably caused by a decrease in the redox potential of the medium.

Kunert [58] investigated 19 sulphur-containing amino acids and their derivatives as sources of sulphur for *M. gypseum*. The best sources were substances known as intermediates of sulphur metabolism or those which occur in natural substrates of dermatophytes, viz. cystine, cysteine, glutathione, cysteic and cysteinesulphinic acids, S-sulphocysteine, lanthionine, taurine and also serine-O-sulphate. Methionine, methionine sulphone and the remaining compounds were moderate to poor sources and S-carboxymethyl cysteine was not utilized at all. Eleven organic sulphur compounds not related to amino acids were poor sulphur sources at best.

The majority of papers on the sulphur metabolism of keratinophilic fungi have dealt with the metabolism of cystine. This compound is a good source of sulphur, but poor as the sole source of nitrogen, because of its quite unsuitable elemental composition, C:N:S ratio [59] and the fact that strongly acidic substances are produced during its metabolism (see below). When added to a suitable medium, cystine is usually inhibitory. In concentrations higher than ca. 1 mM (0.24 mg/ml) it can cause a prolongation of the lag phase, a delay in growth and a decrease in maximum mycelial dry weight [46,59-61]. However, these effects are not so great on rich media, especially on those which contain proteins [48,50,51,54, 55,60,62,63].

Poor growth on many cystine-containing media is not due to an inability of keratinophilic fungi to metabolize cystine. On the contrary, cystine is taken up rapidly from the medium even in the presence of better nutrients. An excess of sulphur in this compound is eliminated as inorganic sulphate excreted into the medium. The oxidation of cystine sulphur to sulphate has been described repeatedly [20,21,27,46,48,54-56,59-68]. It has been confirmed that keratinophilic fungi are adapted to the metabolization of cystine, which proceeds even in the presence of more suitable nutrients. Cystine is used not only as a source of sulphur but also as a source of carbon and nitrogen. A large excess of sulphur (the S content of cystine is 26.7%, while that of dermatophyte dry mycelium only 0.5 to 1.2%, [59]) is eliminated by oxidation to inorganic sulphate excreted into the cultivation fluid. In this way, up to 90% of the original organic sulphur can be converted to sulphate in the medium. Sulphate excretion counteracts an overalkalinization of the environment during growth on substrates which are rich in nitrogen.

In the 1970s we found in our laboratory that dermatophytes growing on media containing free cystine produce not only sulphate, but also the lower oxidation product - sulphite [64,65,69]. Free sulphite was found in some cultures in concentrations of up to 1mg/ml (as $Na_2SO_3$ [65]). However, in most experiments sulphite was found in a bound form. The "bound sulphite" was in fact S-sulphocysteine, which originates in neutral to alkaline solutions by the reaction of sulphite with the cystine remaining in the medium:

$$\underset{\text{cystine}}{\text{cys-SS-cys}} + \underset{\text{sulphite}}{HSO_3^-} \longrightarrow \underset{\text{cysteine}}{\text{cys-SH}} + \underset{\text{S-sulphocysteine}}{\text{cys-SSO}_3^-}$$

(the "sulphitolysis" [70,71]). Sulphite can be liberated from S-sulphocysteine by cyanide according to the following equation:

$$\text{cys-SSO}_3^- + CN^- \longrightarrow \text{cys-SCN} + SO_3^{2-}$$

This reaction was used to determine both free and combined S-sulphocysteine [72].

In experiments with 16 species of dermatophytes and 10 keratinophilic fungi from soil [21,27], the main product of cystine sulphur oxidation was sulphate. However, sulphite was also always produced. Sulphite formation was highest when growth began and on poor media; on media with rapid growth, sulphate was predominant. Sulphite was usually present in the bound form and free sulphite was only found after consumption of cystine from the medium or when sulphitolysis was inhibited by an acidification of the culture fluid. During the growth on wool extract containing cystine combined in proteins, both sulphate and sulphite were also released into the medium [62]. A comparison of various dermatophyte species [20,21] led to the conclusion that general features of cystine metabolism are the same in the whole group. In obligate parasites with limited growth in culture, the oxidation of cystine was, however, slower and/or incomplete. Ten keratinophilic fungi from soil utilized cystine also as a source of carbon and nitrogen in the way that dermatophytes did. However, the differences among individual species were greater in this rather heterogeneous group [27,63].

Of 30 nonkeratinophilic fungi from different taxonomical and ecological groups, 19 showed some ability to utilize cystine not only as a source of sulphur but also as a source of carbon and nitrogen and six strains consumed all the cystine present [67]. Excess sulphur was again excreted into the medium as sulphate and/or sulphite. The use of cystine as a C and N source is therefore not limited to keratinophilic fungi, but in nonkeratinophiles this ability is, on average, much less developed.

Kunert [56,73] investigated the metabolization of a further 10 sulphur amino acids and their derivatives, which were added to the glucose-arginine medium in concentrations of 2.5 or 5 mM (depending on the sulphur content). All compounds were at least partially taken up by the strain of *M. gypseum* and obviously utilized as sources of S, C and N. Except for methionine and its sulphone, the excess sulphur was oxidized by the mycelium and excreted as sulphate or sulphite. The rate and completeness of oxidation was, however, very different in individual compounds. With cysteinesulphinic acid, S-sulphocysteine, djenkolic acid and cysteic acid metabolization was fast and complete. On the other hand, serine-O-sulphate, taurine, lanthionine and particularly homocystine were oxidized only slowly and incompletely and after a long adaptation. In the concentrations used, all compounds more or less inhibited growth. Serine sulphate and particularly methionine and methionine sulphone markedly inhibited the growth and development of cultures. In the last two compounds the excess sulphur was not eliminated by oxidation but by demethiolation, which produced methanthiol and related volatile substances.

Kunert and Trüper [74] tried to elucidate the intracellular pathway of cystine catabolism in dermatophytes. Experiments with labelled ($^{35}S$) cystine indicated that cystine is first reduced to cysteine and then oxidized by cysteine dioxygenase to cysteinesulphinic acid. This acid is de- or trans-aminated to sulphinyl pyruvate, which decomposes spontaneously to pyruvate and sulphite. Most of the latter is further oxidized to sulphate. Cysteinesulphinic acid is also partly converted to taurine which accumulates in the mycelium. Taken together, the pathway of cyst(e)ine catabolism in *M. gypseum* did not differ substantially from that described in other microorganisms and in animals.

For the role of sulphur (cystine) metabolism in keratin degradation, see section entitled 'Keratin degradation'.

## Extracellular enzymes

Of extracellular enzymes of keratinophilic fungi only the proteases and lipases of dermatophytes were studied in greater detail.

### Proteases

The production of secreted proteolytic enzymes was recognized in dermatophytes over a century ago and it has since been described in scores of papers. This review is only able to present a short overview with selected references.

Proteases are released from the mycelium on most cultivation media. The highest activities are always found on media containing proteins, particularly different forms of keratin. The regulation of the synthesis and secretion of proteases is far from clear and this is partly due to the presence of a number of differently regulated enzymes. Some enzymes are secreted constitutively on media without proteins, particularly when the growth begins [24,75,76]. In *T. rubrum* a constitutive production was also found in the stationary phase [77]. In contrast, in the exponential phase the derepression by the lack of C, N or S sources is probably decisive [76,77]. Accordingly, a repression by suitable sources of carbon and nitrogen (glucose, amino acids) was described [29,76,78-80]. However, ammonium ions do not cause any repression. This is regarded as an adaptation to proteolysis in the presence of deamination and ammonia production [78]. In addition to the regulation by repression and derepression, proteins act as inducers and increase the proteolytic activity severalfold [29,60,76, 79,81].

As suggested in many papers, keratinolytic fungi produce a complex of proteolytic enzymes [78,82-89]. In *T. rubrum*, for example, electrophoresis indicated the presence of enzymes with molecular weights of 25, 31, 45, 53, 71, 93 and 124 kDa; three of them were purified to homogeneity [76,84,90]. However, in no fungus can a complete inventory of extracellular proteases be presented.

Proteolytic enzymes of keratinophilic fungi have a broad range of specificity. They can hydrolyze a great many soluble proteins (e.g. casein, gelatin, serum albumin, egg albumin, hemoglobin, myoglobin, cytochrome c) and insoluble proteins (keratin, elastin, collagen, fibrin, laminin, fibronectin etc.). This broad specificity was also demonstrated by studies with low-molecular synthetic substrates [14,76,79,84,86,91-97]. The often described hydrolysis of elastin and collagen [78,79,84,89-91,98-101] is probably only a consequence of the broad specificity of cleavage and does not prove the presence of specialized elastases and collagenases in keratinophilic fungi.

Extracellular enzymes of dermatophytes and soil inhabiting keratinophilic fungi belong to neutral or alkaline proteases. Their optimum pH on various proteins are in the 6 to 9 pH range; with insoluble proteins and particularly keratin, the values usually approach the upper limit. In exceptional cases, optimum values of pH 9 to 10 were found even on soluble proteins [81,92,102]. On the other hand, Tsuboi *et al.* [85,86] and Qin *et al.* [103] isolated an enzyme from *T. mentagrophytes* which attacked human callus optimally at pH 4.5. This enzyme was, however, sensitive to inhibitors of serine proteases and therefore cannot be categorized as an "acid" (aspartyl) protease.

Nearly 20 proteases of keratinophilic fungi were purified to homogeneity [82-84,90,93,94,102-114]. However, their classification within the international system of enzymes remains mostly uncertain. According to their characteristics (e.g. the alkaline optimum pH) the proteases of dermatophytes were described first as trypsin-like and later (according to their specificity) as chymotrypsin-like. Recently purified enzymes are mostly sensitive to class-specific inhibitors of serine proteases. They are often also inhibited by chelating agents (e.g. EDTA), which may be due to the sequestration of $Ca^{2+}$ ions. Calcium ions are frequently bound by serine proteases and increase their activity and stability. Many fungal proteases belong to the class of metalloproteases and consequently, the production of metalloenzymes is also highly probable in keratinophilic fungi. Recent studies have revealed that many fungal "chymotrypsin-like" or "elastase-like" enzymes are in fact members of the subtilisin subfamily of serine proteases [115]. Whether this is also true for enzymes of keratinophiles must be tested in future by molecular genetic studies of their genes.

The secretion of cysteine proteases has not yet been proven with certainty. Inhibitory effects of sulphydryl agents on several enzymes [94,98,104,116] may be due to the blocking of essential cysteines outside the active centre. No aspartyl ("acid") proteases have been found, which corresponds to the alkalinization tendency of keratinophilic fungi.

In many studies of proteolytic enzymes of keratinophiles, the hydrolysis of keratinaceous substrates was also investigated. The measure of keratinolytic activity was usually the release of soluble proteins, peptides and amino acids from these substrates [17,30,78,91,92,103, 105,107,117-131]. To attain a measurable activity the substrate had to be ground or cut to pieces. The most easily accessible substrate was soft keratin (e.g. human plantar callus) which is, however, attacked also by nonspecific proteases [132,133]. In hard keratins the rate of hydrolysis corresponds roughly to the "hardness" i.e. cystine content. Nails, feather or wool are therefore cleaved more easily than human hair. Among mammalian hairs, those with a large medulla are least resistant. In rodents' hair the preferentially hydrolyzed part is medulla, which is not a true keratin. The most popular method for estimating keratinolytic activity, using cut guinea pig hair [105] therefore has some drawbacks. Finely cut human hair is a suitable substrate but the measurable activity could be very low. The use of soluble products of keratin extraction e.g. cytokeratins of callus [84,134,135] or other soluble and reprecipitated derivatives [123,136,137] cannot be recommended, since they are devoid of the resistance to proteolysis of the native solid keratin. Enzymes with keratinolytic activity were also isolated from non-dermatophytes like *Scopulariopsis brevicaulis* [111] and even from two members of Mucorales [109]. Their basic characteristics were quite similar to those of dermatophyte serine proteases.

It is questionable whether the term "keratinase" is appropriate for the proteases of keratinophilic fungi. The latter enzymes surpass the nonspecific proteases (trypsin, papain) in their activity against keratinaceous substrates [105,117,119,124,138]. However, their effect typically declines over time and may even cease [83,117,124, 134,139,140]. In hard keratins, the loss in weight does not exceed the content of non-keratins (5 to 10%) and keratin particles have never been completely dissolved. Complete degradation of the hard keratin is possible only after its denaturation by splitting the disulphide bonds (see section entitled 'Keratin degradation'). Because the proteases of keratinolytic fungi alone are unable to digest the native hard keratin, the term "keratinase" is rather misleading, as stated also by Takiuchi *et al.* [107]. In any case, it should not be used automatically for any protease isolated from a keratino-philic fungus.

In dermatophytes the secretion of proteases into the tissue of the host in the parasitic phase was also investigated. The presence of the above enzymes in the surroundings of hyphae that penetrate the host skin was demonstrated by immunohistochemical methods [110,141-143]. No clear positive correlation was found between proteolytic activity and pathogenicity or virulence. Non-pathogenic, soil keratinophilic fungi were as good producers of proteases as dermatophytes. Moreover, in dermatophytes the proteolytic and keratinolytic activities were not lower in opportunistic species as compared to obligate parasites [14,78,79,135,144-146]. However, some authors did find differences in proteolytic activity among strains isolated from different types of lesions [101,144,147-150]. Strains from acute, inflamed and deep lesions were usually more proteolytic in vitro.

### Lipases and esterases

In dermatophytes, the production of lipases and esterases was demonstrated by plate tests and diagnostic kits [2,14,135,146,151] as well as on liquid media. The most thorough studies are those of Böhme [8,9,152-154], Hellgren & Vincent [10,155] and Das & Banerjee [12]. The best natural substrates for secreted lipases were olive oil and oleum pedum tauri. Triolein and Tweens (particularly Tweens 60 and 80) were also hydrolyzed. Cleaving tributyrin [8,153] indicated the presence of esterases and the activity of phospholipase A (hydrolyzing lecithin) was also demonstrated [12,13,135,156]. Brasch and Zaldua [14] included *Scopulariopsis brevicaulis* in their study; the production of lipases in this fungus was poorer than in dermatophytes. There are usually great differences in lipolytic activity between various species and strains of dermatophytes. *T. rubrum* seems to be particularly weak in this respect [10,154]. Lipases and phospholipases are not only produced on media containing lipids but also on media with keratin (human hair, callus) [14] and even on Sabouraud glucose-peptone broth (phospholipase A) [12].

### Other enzymes

The dermatophytes produce an extracellular phosphatase with a broad specificity and optimum pH around 8.7 [14,96,97,119,157]. The enzyme is probably secreted constitutively as it was found also on media containing inorganic phosphate. For the production of amylase, see section entitled 'Carbon metabolism' (utilization of starch). In experiments using diagnostic kits for semiquantitative determination of enzymic activities [14,96,97], the presence of various extracellular glycosidases was demonstrated. However, their role in the use of glycides as nutrients has yet to be described.

## Keratin degradation

The light and electron microscopic studies into the micromorphology of keratin degradation by fungi concluded that degradation is an enzymic process. Many authors then investigated the physiology and biochemistry of keratinolysis in cultures of keratinophilic fungi on keratinaceous substrates in liquid media [7,17,30,48,59,60,64, 72,80,92,117,120-123,126-131, 139,140,158-174]. The most frequently used substrates were human hair and sheep wool, but hair from guinea pigs and other mammals, feather, ground horn and hoof, human plantar callus and even snake skin [131] were also used. Keratin was sterilized by mild autoclaving or by ethylene oxide, and also but less often by organic solvents, solutions of antibiotics or by "tyndalization". In the author's experience a number of sterilization methods produce similar results, provided there is no serious denaturation, which can be caused by prolonged autoclaving or autoclaving the substrate immersed in a liquid medium. Fine grinding and particularly ball milling the keratinaceous substrates also cause denaturation [139]. Partially denatured substrate is degraded faster and specialized structures such as "perforating organs" or "boring hyphae" become rare or disappear (Kunert, nonpublished data). Defatting keratinaceous substrates by organic solvents removes inhibitory components of sebum but may damage the structure of the tissue.

Keratinophilic fungi can grow on keratinaceous substrates in distilled water, but grow better in solutions of salts. Decomposition is particularly stimulated by inorganic phosphate (0.05 to 0.1%). Small concentrations (0.1%) of glucose and also supplementation with amino acids, peptone or yeast extract may accelerate the beginnings of growth. Higher concentrations of soluble nutrients, however, inhibit the attack on the substrate [126,140,145,158,161,162,164,171,172]. Since mycelium and the remaining insoluble substrate cannot be separated, quantitative measurement of keratin degradation poses a problem. Usually only the loss of "total dry weight" (keratin + mycelium) is given. Ziegler and Böhme [158] calculated the net loss of weight of the substrate by the "economic coefficient" of 3.5 (1 mg mycelial dry weight formed from 3.5 mg digested substrate). Chesters and Mathison [140] dissolved the attacked substrate in hot NaOH and measured the residual dry weight (alkali-insoluble remnants of mycelial cell walls). For the conversion of residual dry weight to the original one, coefficients estimated by further experiments were used. As the solubility of mycelia in alkali changes substantially with age, even this method must be regarded an approximation.

In cultures on keratin in a liquid medium the digestion of 30% to 50% of the substrate is common even with hard keratin and losses of total dry weight surpassing 80% were recorded. Microscopic observation usually showed that large parts of the substrate had been completely digested. The limits of degradation are therefore not due to the presence of a fraction of the substrate not accessible to the fungus, but rather to the accumulation of metabolites and/or consumption of some nutrients in older cultures. In weakly keratinolytic fungi the degradation does not usually exceed 40% even after 8 and more weeks. With losses of weight below 20% the fungus may not be truly keratinolytic and may digest only non-keratins, representing 5% to 10% even of the hard keratins. The rate and completeness of the degradation is, of course, dependent on the kind of substrate and correspond roughly to its hardness, viz. cystine content. Human and also dog, horse and cattle hairs are therefore attacked more slowly than rodents' hair, sheep wool or feather. Nail and horn are little resistant. Soft keratin (e.g. callus) contains large amounts of non-keratins and is attacked and degraded also by non-keratinolytic fungi.

The results of the studies cited above suggest that strongly keratinolytic fungi, weakly keratinolytic fungi and those digesting obviously only non-keratins do not represent clearly separated groups. Evidently keratinolytic strains were found not only among members of the order Onygenales, but also in other taxonomic groups, even in the Mucorales [30,109,111,127,129,131,172]. It is conceivable that moderately and negligibly keratinolytic strains exist within one species. In *Scopulariopsis brevicaulis*, for example, some authors described a keratinolytic ability [7,111,128,145,172] while others could not find it [167,175].

Intensity of keratin degradation in liquid media is correlated to some extent with its micromorphology.

Fungi forming numerous perforating organs usually cause a fast substrate degradation. However, some strains attacking the substrate from the surface without perforating organs may also cause high losses of substrate dry weight. Species forming only long and thin "boring hyphae" are poor decomposers, whereas wide (swollen) boring hyphae suggest a stronger keratinolytic ability [167; see also 176].

Keratin degradation in a liquid medium is accompanied by the release of cleavage products into the cultivation fluid. Peptides with molecular weights of 1 to 2 kDa are the prevalent fraction, but free amino acids and high molecular proteins are also present [166]. The concentration of all products of protein hydrolysis may reach hundreds of micrograms per ml, but it is only weakly correlated with the degree of substrate degradation [167]. Even poorly keratinolytic fungi may accumulate high amounts of peptidic compounds in the medium. A better indicator of keratinolysis is the rise in pH, reflecting the utilization of keratin proteins, deamination, and ammonia production (see section entitled 'Sources and metabolism of nitrogen'). All keratinolytic microorganisms raise the pH to at least 8 [177]. In cultures of keratinolytic fungi on keratin the pH usually peaks at 8 to 9; values exceeding 10 were exceptionally reported [140]. However, the meaning of pH values is lessened by the volatility of ammonia that sets an upper pH limit common to all fungi.

In the cultivation fluid sulphur-containing metabolites are also accumulated. Keratin is very rich in sulphur (ca. 0.5% in soft keratins and up to 4% in the hard tissues). Excess sulphur is eliminated by oxidation to sulphate excreted into the culture fluid [48,59,60,68,72,92, 128-131,145,162,167,169]. Concentrations exceeding 1 mg/ml (as $Na_2SO_4$) were measured. Sulphate is a final and rather inert metabolite and its concentration in the medium is a good measure of substrate degradation attained [167]. Inorganic thiosulphate was found in the medium by some authors [128-131] by a colorimetric method, but its presence could not be demonstrated by analytical isotachophoresis [68]. A positive interference with the colorimetric method thus cannot be excluded.

In comparison with sulphate the concentrations of organic sulphur compounds are relatively low. The main fraction is represented by cystine- and S-sulphocysteine-containing peptides [166]. Published data on the content of cysteine (thiols) in the cultivation fluid are rather contradictory. Whereas some authors measured tens of micrograms per ml cysteine [122,127-130,160,164] others could find only small to trace amounts of thiols [48,68,72,92, 130,158,162,163,165,167-170]. It must be borne in mind that thiols including cysteine are, in an alkaline milieu, easily reoxidized by air oxygen to disulphides.

As already discussed in the previous chapter, the effect of proteolytic enzymes ("keratinases") on native hard keratin is rather limited and keratinolysis cannot be regarded as a purely proteolytic attack. In other keratinolytic organisms, as, for example, wool-eating larvae of some insects [178] or streptomycetes [68,179] it was revealed that prior to proteolysis the substrate is denatured by the reduction of disulphide bridges. However, in keratinolytic fungi the reduction of keratin by extracellular enzymes and/or by the excretion of thiols could not be demonstrated. Similarly, the alkalinization of the medium, regarded as the main denaturing factor by Chesters & Mathison [140] and Ziegler & Böhme [16] is alone probably not sufficient for rendering hard keratin cleavable by fungal proteases [124].

As discussed in section entitled 'Sources and metabolism of sulphur', keratinolytic fungi oxidize cystine sulphur not only to sulphate, but also to sulphite that reacts with cystine giving rise to cysteine and S-sulphocysteine. Since sulphitolysis of disulphide bridges takes place also in cystine combined in proteins including keratin, another hypothesis on the biochemical mechanism of keratin degradation by fungi was presented [64]. This hypothesis postulates that sulphitolysis is the key reaction of keratinolysis. Fungi excrete sulphite and ammonia and, in an alkaline milieu, they cleave disulphide bonds of the substrate by sulphite. In this way the substrate is gradually denatured and rendered susceptible to the attack by fungal proteases. Further investigations lend support to the above hypothesis. S-sulpho groups ($R\text{-}SSO_3H$) were demonstrated in human hairs attacked by *M. gypseum in vitro* by means of histochemical methods [69] and their presence in the degraded substrate confirmed later by other authors [168,171,173,180]. The main product of sulphitolysis, S-sulphocysteine, was found in the cultivation fluid of cultures of keratinolytic fungi on keratin as free and also combined in peptides [68,72,166,167,181]. In older cultures, as much as 80% of organic sulphur in the cultivation fluid was present as S-sulphocysteine [72]. Concentrations of S-sulphocysteine correlated at the same time with the degree of substrate degradation attained by various fungi [167] and in fungi without a keratinolytic ability the signs of sulphitolysis were also missing [167,171,173]. It was also demonstrated that sulphite strongly stimulated the proteases of *M. gypseum* in the hydrolysis of wool keratin. Sulphitolyzed sheep wool could be completely solubilized by the above enzymes [125].

Experimental data thus confirmed the hypothesis on sulphitolysis as the key reaction of keratinolysis in fungi. Keratin degradation is therefore most probably the result of the action of three factors: deamination (creating an alkaline environment needed for substrate swelling, sulphitolysis and proteolytic attack), sulphitolysis (denaturing the substrate by removing its disulphide bridges) and proteolysis (cleaving the denatured substrate to soluble products). These factors are at least partially present also in non-keratinolytic fungi, but only in keratinolytic species they were developed to an extreme and were concerted in action. For more details on biochemical aspects of keratinolysis the reader is referred to a recent review [182].

In dermatophytes, keratinolytic abilities were compared in species differing in the degree of adaptation to parasitism [120,145,158,161,167]. The results revealed that many obligate parasites (particularly the anthropophilic species) degraded the hard keratin rather slowly. This is, however, probably caused only by their overall slow growth and poor metabolic performance in vitro. It is generally accepted that the ability to decompose keratin of the epidermis and its adnexes and to utilize it as a main source of nutrition is a precondition of parasitism in the keratinized tissues of the host. However, there are many strongly keratinolytic fungi (including some members of the dermatophyte genera *Microsporum* and *Trichophyton*) that are almost never found as parasites. In the parasitic species, keratinolytic ability must be therefore accompanied by other adaptations to conditions prevailing in the epidermis. The fungus here encounters supraoptimal temperature, low water potential, microaerobic conditions, carbon dioxide production, components of sweat and sebum and what is the most important, the factors of nonspecific and specific immunity that encompass even the dead layers on the body surface. Presumed adaptations and virulence factors of dermatophytes remain, however, little understood [see 183-186].

## References


1. Philpot CM. The use of nutritional tests for the differentiation of dermatophytes. Sabouraudia 1977;15: 141-150.
2. Calvo MA, Trape J, Abarca L, Cabanes FJ, Calvo RM, Bruguera T. Variability of biochemical characteristics in strains of *Trichophyton mentagrophytes.* Mycopathologia 1986; 93: 137-140.
3. Ziegler H. Untersuchungen über die Wirkung von Griseofulvin auf *Microsporum canis.* Z Allg Mikrobiol 1963;3: 211-224.
4. Ziegler H, Böhme H. Die Verwertung von Amylum solubile als Hauptkohlenstoffquelle durch Dermatophyten und Schimmelpilze. Dermatol Monatsschrift 1970;156: 52-66.
5. Kushwaha RKS, Agrawal SC. Amylolytic capability of some keratinophilic fungi. Proc Nat Acad Sci India 1975; 45: 182-184.
6. Kushwaha RKS, Agrawal SC. Carbon metabolism in some soil inhabiting keratinophilic fungi. Trans Mycol Soc Japan 1977;18: 47-56.
7. Benedicto T. Contribution à l'étude du *Scopulariopsis brevicaulis* Bainier. Recherche de conditions optimales de croissance, activité protéolytique et kératinolytique in vitro. Thèse de Doctorat, Faculté des Sciences, Toulouse 1973.
8. Böhme H. Über die Verwertung von Lipiden und die Ausscheidung lipolytischer Enzyme durch *Microsporum gypseum.* Zeitschrift für Haut- und Geschlechtskrankheiten 1968;43: 1017-1023.
9. Böhme H, Schmollack E. Abbau von Vernix caseosa durch *Microsporum gypseum* (Bodin) Guiart et Grigoraki in vitro. Dermatol Monatsschrift 1969; 155: 171-177.
10. Hellgren L, Vincent J. Lipolytic activities of some dermatophytes. J Med Microbiol 1980;13: 155-158.
11. Mathison EG. Some aspects of the physiology of *Keratinomyces ajelloi* Vanbreuseghem. Trans Brit Mycol Soc 1962;45: 430-431.
12. Das SK, Banerjee AB. Lipolytic enzymes of *Trichophyton rubrum.* Sabouraudia 1977;15: 313-323.
13. Das SK, Banerjee AB. Effect of undecanoic acid on the production of exocellular lipolytic and keratinolytic enzymes by undecanoic acid sensitive and resistant strains of *Trichophyton rubrum.* Sabouraudia 1982;20: 175-184.
14. Brasch J, Zaldua M. Enzyme patterns of dermatophytes. Mycoses 1994;37: 11-16.
15. Garg AP, Müller J. Fungitoxicity of fatty acids against dermatophytes. Mycoses 1993;36: 51-63.
16. Ziegler H, Böhme H. Über die aktive Alkalisierung des Terrains als physiologisches Prinzip bei der Keratinolyse durch Dermatophyten. Dermatol Monatsschrift 1969;155: 178-181.
17. Böhme H, Ziegler H. Keratinabbau durch Pilze. Arch Mikrobiol 1967;57: 93-110.
18. Paveia MH. Culture medium alkalinization by dermatophytes. Mycopathologia 1975;55: 35-40.
19. Danew P, Klossek P. Zur Abhängigkeit physiologischer Eigenschaften von *Trichophyton mentagrophytes* vom pH-Wert des Mediums. Mycoses 1989;32: 303-308.
20. Kunert J. Utilization of cystine by dermatophytes on a gelatin medium. Folia Microbiol 1988;33: 121-128.
21. Kunert J. Utilization of cystine by dermatophytes on glucose-peptone media. Folia Microbiol 1988;33: 188-197.
22. Refai M, Rieth H. Änderung des pH-Wertes der Nährböden durch Dermatophyten. Zbl Bakt I Orig 1964;194: 114-120.
23. Burack AM, Knight SG. Observation on submerged growth and deamination of amino acids by dermatophytes. J Invest Dermatol 1958; 30, 197-199.
24. Ziegler H, Böhme H. Untersuchungen über den Stoffwechsel der Dermatomyzeten. Mykosen 1962;5: 1-17.
25. Rippon JW, LeBeau LJ. Germination and initial growth of *Microsporum audouinii* from infected hairs. Mycopathologia 1965;26: 273-288.
26. Kushwaha RKS. Physiological studies of some common Indian keratinophilic molds. Proc Nat Acad Sci India 1981;51: 105-106.
27. Kunert J. Utilization of L-cystine by soil keratinophilic fungi. Acta Univ Olomuc Fac Med 1988;120: 11-22
28 Braun W, Lieschke E. Beziehungen der Wasserstoffionenkonzentration der Haut zur Infektionsmöglichkeit mit Trichophytie. Dermatol Monatsschrift 1969;155: 651-660.
29. Ziegler H, Böhme H. Über den Stoffwechsel der Gattung *Microsporum* Gruby. Arch Klin Exper Dermatol 1964;218: 611-631.
30. Ziegler H. Vergleichende Untersuchungen über den Stoffwechsel von Schimmelpilzen und Dermatophyten. Z Allg Mikrobiol 1966;6: 74-85.
31. Ghosh GR. Comparative physiology of some representatives of the Gymnoascaceae. Mycopathologia 1960;13: 161-180.
32. Chattaway FW, Toothill C, Barlow AJE. The amino acid metabolism of *Microsporum canis.* J Gen Microbiol 1962;28: 721-732.
33. Kuehn HH. Nutritional experiments on *Arthroderma tuberculatum.* Mycopathologia 1961;14: 123-128.
34. Kuehn HH. A preliminary study of the nutritional requirements of *Myxotrichum uncinatum.* Mycopathologia 1962;16: 222-228.
35. Ragot J. Influence de la nutrition azotée sur la croissance, la sporulation et la morphologie du *Keratinomyces ajelloi* Vanbr. C R Soc Biol (Paris) 1966;160: 682-686.
36. Zussman RA, Vicher EE, Lyon I. Aromatic amino acid biosynthesis in *Trichophyton rubrum.* III. Absence of the shikimic acid pathway. Mycopathologia 1970;42: 1-8.
37. Clérivet A, Ragot J. Contribution a l étude des exigences nutritives du *Trichophyton verrucosum.* C R Acad Sci (Paris) D 1971;272: 1772-1775.
38. Dujardin L, Biguet J, Lacoste L. Croissance et reproduction sexuée du champignon dermatophyte *Arthroderma vanbreuseghemii* Takashio (*Trichophyton mentagrophytes* Robin Blanchard) sur un milieu de culture synthétique. Mycopathologia 1975;57: 113-120.
39. Kolár Z, Kunert J. Verwertung verschiedener Aminosäuren als Stickstoffquellen für das Wachstum des Dermatophyten *Microsporum gypseum.* Acta Univ Olomuc Fac Med 1977;81: 95-104.
40. Hilger AE, Lawrie MJ, Wilson DA. Growth of *Trichophyton mentagrophytes* on individual amino acids. Sabouraudia 1979;17: 299-303.
41. Danew P, Marquardt I, Friedrich E. Aurich H. Aminosäureassimilation und -sekretion bei Dermatophyten I. Mykosen 1980; 23: 632-639.
42. Singh KV, Agrawal SC. Nutritional requirements of keratinophilic fungi and dermatophytes for conidial germination. Mycopathologia 1982;80: 27-32.
43. Rosenthal SA, Sokolsky H. Enzymatic studies with pathogenic fungi. Dermatol Int 1965;4: 72-79.
44. Kunert J, Lenhart K. Studies on the variability of dermatophytes. III. Physiological characteristic of *Trichophyton rubrum* strains. Acta Univ Olomuc Fac Med 1979;89: 23-34.
45. Kunert J, Lenhart K. Studies on the variability of dermatophytes. VI. Physiological characteristic of *Trichophyton mentagrophytes* strains. Acta Univ Olomuc Fac Med 1981;99: 73-83.
46. Kunert J. Cystine metabolism in the dermatophyte *Microsporum gypseum.* I. Utilization of cystine in media containing glucose. Acta Univ Olomuc Fac Med 1985;108: 39-52.
47. Ziegler H. Untersuchungen über die stoffwechselphysiologische Wirkung verschiedener Kohlenhydrat- und Stickstoffquellen auf *Microsporum canis.* Mykosen 1961;4: 39-57.
48. Stahl WH, McQue B, Mandels GR, Siu RGH. Studies on the microbiological degradation of wool. I. Sulphur metabolism. Arch Biochem 1949;20: 422-432.
49. Kunert J. Inorganic sulphur sources for the growth of the dermatophyte *Microsporum gypseum.* Folia Microbiol 1981;26: 196-200.
50. Zussman RA, Vicher EE, Lyon I. Nutritional and environmental factors affecting pigmentation in *Trichophyton rubrum.* Mycopathologia 1961;14: 205-214.
51. Raubitschek F. Organic sulphur and the keratinophilia of dermatophytes. Sabouraudia 1962;2: 40-42.
52. Koehne GW. Nutrition of three species of *Microsporum.* Mycopathologia 1962;18: 199-206.
53. Davidsson M, Unestam T. Factors affecting the sexual reproduction of a dermatophyte, *Arthroderma benhamiae*, in synthetic media. Physiol Plantarum 1974;31: 237-244.
54. Danew P, Marquardt I, Friedrich E, Aurich H. Aminosäureassimilation und -sekretion bei Dermatophyten II. Mykosen 1981;24: 561-565.
55. Danew P, Friedrich E, Farr W, Aurich H, Haschen RJ. Abbau und Assimilation von Glukose und Aminosäuren durch *Microsporum gypseum.* Mykosen 1982;25: 143-150.
56. Kunert J. Metabolism of sulfur-containing amino acids in the dermatophyte *Microsporum gypseum.* I. Neutral amino acids. J Basic Microbiol 1985;25: 31-37.
57. Rippon JW, Scherr GH. Induced dimorphism in dermatophytes. Mycologia 1959;51: 902-914.
58. Kunert J. Organic sulphur sources for the growth of the dermatophyte *Microsporum gypseum.* Folia Microbiol 1981;26: 201-206.
59. Ziegler H. Reichmann G. Über den Schweffelstoffwechsel von *Microsporum canis.* Mykosen 1968;11: 903-907.
60. Ziegler H, Böhme H, Reichmann G. Stoffwechselphysiologische Untersuchungen über den Abbau von Proteinen durch *Microsporum gypseum* und *M. canis.* Dermatol Monatsschrift 1969;155: 835-856.
61. Ziegler H, Böhme H. Biologie und Chemie der Keratine und der Keratinisation sowie des Keratinabbaues durch vorzugsweise pathogene Pilze. Biol Rundschau 1977;15: 27-40.
62. Kunert J. Cystine metabolism in the dermatophyte *Microsporum gypseum.* II. Utilization of cystine in media containing peptides and proteins. Acta Univ Olomuc Fac Med 1985;111: 37-47.
63. Malviya HK, Hasija SK, Rajak RC. In vitro utilization of cystine by keratinophilic fungi inhabiting a gelatin factory. Mycopathologia 1992;118, 147-152.
64. Kunert J. Keratin decomposition by dermatophytes: evidence of the sulphitolysis of the protein. Experientia 1972;28: 1025-1026.
65. Kunert J. Formation of sulphate, sulphite and S-sulphocysteine by the fungus *Microsporum gypseum* during growth on cystine. Folia Microbiol 1975;20: 142-151.
66. Kunert J. Utilization of various concentrations of free cystine by the fungus *Microsporum gypseum.* J Basic Microbiol 1987;27: 207-213.
67. Kunert J. Utilization of L-cystine as a source of carbon and nitrogen by various fungi. Acta Univ Olomuc Fac Med 1989;123: 351-364.

68. Kunert J. Biochemical mechanism of keratin degradation by the actinomycete *Streptomyces fradiae* and the fungus *Microsporum gypseum*: a comparison. J Basic Microbiol 1989;29: 597-604.
69. Kunert J. Thiosulphate esters in keratin attacked by dermatophytes in vitro. Sabouraudia 1972;10: 6-13.
70. Milligan B Swan, JM. Bunte salts (R.SSO3Na). Rev Pure Appl Chem 1962;12: 72-94.
71. Kella NKD, Kang YJ, Kinsella JE. Effect of oxidative sulfitolysis of disulfide bonds of bovine serum albumin on its structural properties: a physicochemical study. J Protein Chem 1988;7: 535-548.
72. Kunert J. Keratin decomposition by dermatophytes. I. Sulfite production as a possible way of substrate denaturation. Z Allg Mikrobiol 1973;13: 489-498.
73. Kunert J. Metabolism of sulfur-containing amino acids in the dermatophyte *Microsporum gypseum*. II. Acidic amino acid derivatives. J Basic Microbiol 1985;25: 111-118.
74. Kunert J, Trüper HG. Cystine catabolism in mycelia of *Microsporum gypseum*, a dermatophytic fungus. Arch Microbiol 1986;145: 181-186.
75. Danew P, Friedrich E. Untersuchungen zur Peptidaseaktivität hautpathogener Pilze IV. Mykosen 1980;23: 502-511.
76. Apodaca G, McKerrow JH. Regulation of *Trichophyton rubrum* proteolytic activity. Infect Immun 1989;57: 3081-3090.
77. Apodaca G, McKerrow JH. Expression of proteolytic activity by cultures of *Trichophyton rubrum*. J Med Vet Mycol 1990;28: 159-171.
78. Meevootisom V, Niederpruem DJ. Control of exocellular proteases in dermatophytes and especially *Trichophyton rubrum*. Sabouraudia 1979;17: 91-106.
79. Grzywnowicz G, Lobarzewski J, Wawrzkiewicz K, Wolski T. Comparative characterization of proteolytic enzymes from *Trichophyton gallinae* and *T. verrucosum*. J Med Vet Mycol 1989;27: 319-328.
80. Singh C.J. Characterization of an extracellular keratinase of *Trichophyton simii* and its role in keratin degradation. Mycopathologia 1997;137: 13-16.
81. O´Sullivan J, Mathison GE. The localization and secretion of a proteolytic enzyme complex by the dermatophytic fungus *Microsporum canis*. J Gen Microbiol 1971;68: 319-326.
82. Rippon JW. Extracellular collagenase from *Trichophyton schoenleinii*. J Bacteriol 1968;95: 43-46.
83. Yu RJ, Harmon SR, Grappel SF, Blank F. Two cell-bound keratinases of *Trichophyton mentagrophytes*. J Invest Dermatol 1971;56: 27-32.
84. Asahi M, Lindquist R, Fukuyama K, Apodaca G, Epstein WL, McKerrow JH. Purification and characterization of major extracellular proteinases from *Trichophyton rubrum*. Biochem J 1985; 232: 139-144.
85. Tsuboi R, Ko I-J, Matsuda K, Ogawa H. A ew keratinolytic proteinase from clinical isolates of *Trichophyton mentagrophytes*. J Dermatol 1987; 14: 506-508.
86. Tsuboi R, Ko I-J, Takamori K, Ogawa H. Isolation of a keratinolytic proteinase from *Trichophyton mentagrophytes* with enzymatic activity at acidic pH. Infect Immun 1989;57: 3479-3483.
87. Lobarzewski J, Grzywnowicz K, Wawrzkiewicz K, Staszczak M, Wolski T. Feather keratin as a ligand in an affinity chromatographic technique for isolation of protease from *Trichophyton verrucosum*. J Chromatogr 1990;520: 223-237.
88. Siesenop U, Böhm KH. Comparative studies on keratinase production of *Trichophyton mentagrophytes* strains of animal origin. Mycoses 1995;38: 205-209.
89. Simpanya MF, Baxter M. Multiple proteinases from two *Microsporum* species. J Med Vet Mycol 1996;34: 31-36.
90. Apodaca G, McKerrow, JH. Purification and characterization of a 27,000-Mr extracellular proteinase from *Trichophyton rubrum*. Infect Immun 1989;57; 3072-3080.
91. Yu RJ, Harmon SR, Wachter PE, Blank F. Amino acid composition and specificity of a keratinase of *Trichophyton mentagrophytes*. Arch Biochem Biophys 1969;135: 363-370.
92. Clérivet A, Ragot J. Activité kératinolytique et protéolytique du *Trichophyton verrucosum* cultivé sur la laine Merino non dénaturée. C R Acad Sci (Paris) D 1974;278: 3191-3194.
93. Ruffin P, van Brussel E, Biguet J, Biserte G. Partial characterization of two extracellular aminopeptidases (arylamidases) from the dermatophyte *Keratinomyces ajelloi*. Biochimie 1979;61: 495-500.
94. Sanyal AK, Das SK, Banerjee AB. Purification and partial characterization of an extracellular proteinase from *Trichophyton rubrum*. Sabouraudia 1985;23: 165-178.
95. Kunert J, Kasafírek E. Preliminary characterization of extracellular proteolytic enzymes of dermatophytes by chromogenic substrates. J Med Vet Mycol 1988;26: 187-194.
96. Brasch J, Martins B-S, Christophers E. Enzyme release by *Trichophyton rubrum* depends on nutritional conditions. Mycoses 1991;34: 365-368.
97. Brasch J, Fölster-Holst R, Christophers E. Tinea durch *Trichophyton equinum*. Hautarzt 1998;49: 397-402.
98. Rippon JW, Varadi DP. The elastases of pathogenic fungi and actinomycetes. J Invest Dermatol 1968;50: 54-58.
99. Weitzman I, Gordon MA, Rosenthal SA. Determination of the perfect state, mating type and elastase activity of the *Microsporum gypseum* complex. J Invest Dermatol 1971;57: 278-282.
100. Hopsu-Havu VK, Tunnela E. Production of elastase, urease and sulphatase by *Epidermophyton floccosum*. Mykosen 1977;20: 91-96.
101. Wawrzkiewicz K, Rubaj B, Ziolkowska G. Elastolytical activity in vitro and in vivo of the mycelial and spore forms of the *Trichophyton verrucosum* strains. Mykosen 1978;21: 236-244.
102. Day WC, Toncic P, Stratman SL, Leeman U, Harmon SR. Isolation and properties of an extracellular protease of *Trichophyton granulosum*. Biochim Biophys Acta 1968;167: 597-606.
103. Qin L-M, Dekio S, Jidoi J. Some biochemical characteristics of a partially purified extracellular keratinase from *Trichophyton schoenleinii*. Zbl Bakt I Orig 1992;277: 236-244.
104. Roberts FF, Doetsch RN. Purification of a highly active protease from a *Microsporum* species. Antonie van Leeuwenhoek 1967;33: 145-152.
105. Yu RJ, Harmon SR, Blank F. Isolation and purification of an extracellular keratinase of *Trichophyton mentagrophytes*. J Bacteriol 1968;96: 1435-1436.
106. Leighton TJ, Stock JJ. Biochemical changes during fungal sporulation and spore germination. I. PMSF inhibition of macroconidial germination in *Microsporum gypseum*. J Bacteriol 1970;101: 931-940.
107. Takiuchi I, Higuchi D, Sei Y, Koga M. Isolation of an extracellular proteinase (keratinase) from *Microsporum canis*. Sabouraudia 1982;20: 281-288.
108. Lee KH, Park KK, Park SH, Lee JB. Isolation, purification and characterization of a keratinolytic proteinase from *Microsporum canis*. Yonsei Med J (Korea) 1987;28: 131-138.
109. Rajak RC, Malviya H, Deshpande H, Hasija SK. Production and comparative characterization of keratinases from *Absidia cylindrospora* and *Rhizomucor pusillus*. Indian J Microbiol 1991;31: 243-250.
110. Sei Y, Ogura Y, Higuchi D, Takiuchi I. Biochemical and immunological studies of neutral protease of *Microsporum canis* - localization and extraction in vivo. Jap J Med Mycol 1992;33, 137-145.
111. Malviya H, Rajak RC, Hasija SK. Purification and partial characterization of two extracellular keratinases of *Scopulariopsis brevicaulis*. Mycopathologia 1992;119: 161-165.
112. Lambkin I, Hamilton AJ, Hay RJ. Partial purification and characterization of a 235.000 Mr extracellular proteinase from *Trichophyton rubrum*. Mycoses 1994;37: 85-92.
113. Ferreiro L. Etiopathogénie des infections a *Microsporum canis*. Étude d une exoprotéase kératinolytique. These de Doctorat, Université de Paris XII. J Mycol Méd 1997;7: 63.
114. Aubaid AH, Muhsin TM. Partial purification and kinetic studies of exocellular proteinase from *Trichophyton mentagrophytes* var. erinacei. Mycoses 1998;41: 163-168.
115. Öhman J, Ek B, Rask L, Tunlid A. Sequence analysis and recognition of a gene encoding a cuticle-degrading serine protease from the nematophagous fungus *Arthrobotrys oligospora*. Microbiology - UK 1996;142: 1605-1616.
116. Higuchi D, Takiuchi I. Inhibitors of the purified extracellular keratinase of a *Microsporum gypseum* strain. Jap J Med Mycol 1980;21: 101-108.
117. Stahl WH, McQue B, Siu RGH. Studies on the microbiological degradation of wool. II. Nitrogen metabolism. Arch Biochem 1950;27: 211-220.
118. Ziegler H. Die Ekto-Enzyme der Dermatomyzeten. I. Hemmungsanalysen. Dermatol Wochenschrift 1965;151: 577-593.
119. Ziegler H. Die Ektoenzyme der Dermatophyten. II. pH-Abhängigkeit und Substratspezifität. Arch Klin Exp Dermatol 1966;226: 282-299.
120. Ziegler H. Vergleichende Untersuchungen über den Stoffwechsel von Schimmelpilzen und Dermatophyten. 2. Keratinabbau. Z Allg Mikrobiol 1967;7: 53-75.
121. Ziegler H. Über den Stoffwechsel der Gattung *Microsporum* Gruby. 2. Abbau von Hornkeratin. Mykosen 1967;10: 539-550 & 559-570.
122. Weary PE, Canby CM. Further observations on the keratinolytic activity of *Trichophyton schoenleini* and *T. rubrum*. J Invest Dermatol 1969;53: 58-63.
123. Page WJ, Stock JJ. Phosphate-mediated alteration of the *Microsporum gypseum* germination protease specificity for substrate: enhanced keratinase activity. J Bacteriol 1974;117: 422-431.
124. Kunert J. Untersuchungen über die proteolytischen Ektoenzyme des Dermatophyten *Microsporum gypseum*. II. Keratinolytische Aktivität der Kultivierungsflüssigkeit. Acta Univ Olomuc Fac Med 1976;79: 125-140.
125. Kunert J. Effect of reducing agents on proteolytic and keratinolytic activity of enzymes of *Microsporum gypseum*. Mycoses 1992;35: 343-348.
126. Kushwaha RKS. The in vitro degradation of peacock feathers by some fungi. Mykosen 1983;26: 324-326.
127. Hasija SK, Malviya H, Rajak RC. Keratinolytic ability of some fungi isolated from gelatin factory campus, Jabalpur. Proc Natl Acad Sci India 1990;60:305-309.
128. Malviya H, Rajak RC, Hasija SK. Keratin degradation by *Scopulariopsis brevicaulis*. J Indian Bot Soc 1991;70: 213-216.
129. Malviya HK, Parwekar S, Rajak RC, Hasija SK. Evaluation of keratinolytic potential of some fungal isolates from gelatin factory campus. Indian J Exper Biol 1992;30: 103-106.
130. Rajak RC, Parwekar S, Malviya H, Hasija SK. Keratin degradation by fungi isolated from the grounds of a gelatin factory in Jabalpur, India. Mycopathologia 1991;114: 83-87.
131. Rajak RC, Malviya HK, Deshpande H, Hasija SK. Keratinolysis by *Absidia cylindrospora* and *Rhizomucor pusillus*: biochemical proof. Mycopathologia 1992;118: 109-114.
132. Bjelland S, Hjelmeland K, Volden G. Degradation of human epidermal keratin by cod trypsin and extracts of fish intestines. Arch Dermatol Res 1989;280: 469-473.
133. Imai S. The effect of the proteolytic enzyme savinase on human plantar skin in vitro. Arch Dermatol Res 1991; 283: 377-381.

134. Takiuchi I, Sei Y, Takagi H, Negi M. Partial characterization of the extracellular keratinase from *Microsporum canis*. Sabouraudia 1984;22: 219-224.
135. Muhsin TM, Aubaid AH, Al-Duboon AH. Extracellular enzyme activities of dermatophytes and yeast isolates on solid media. Mycoses 1997;40: 465-469.
136. Wawrzkiewicz K, Lobarzewski J, Wolski T. Intracellular keratinase of *Trichophyton gallinae*. J Med Vet Mycol 1987;25: 261-268.
137. Wawrzkiewicz K, Wolski T. Lobarzewski J. Screening of the keratinolytic activity of dermatophytes in vitro. Mycopathologia 1991;114: 1-8.
138. Chattaway FW, Ellis DA, Barlow AJE. Peptidases of dermatophytes. J Invest Dermatol 1963;41: 31-37.
139. Stahl WH, McQue B, Mandels GR, Siu RGH. Studies on the microbiological degradation of wool. Digestion of normal and modified fibrillar proteins. Textile Res J 1950;20: 570-579.
140. Chesters CGC, Mathison GE. The decomposition of wool keratin by *Keratinomyces ajelloi*. Sabouraudia 1963;2: 225-237.
141. Grappel SF, Blank F. Role of keratinases in dermatophytosis. I. Immune response of guinea pigs infected with *Trichophyton mentagrophytes* and guinea pigs immunized with keratinases. Dermatologica 1972;145: 245-255.
142. Collins J-P, Grappel SF, Blank F. Role of keratinases in dermatophytosis. II. Fluorescent antibody studies with keratinase II of *Trichophyton mentagrophytes*. Dermatologica 1973;146: 95-100.
143. Lee KH, Park HW, Lee JB. Detection of keratinolytic proteinase in skin tissues from guinea pigs infected with *Microsporum canis* by an immunoperoxidase technique. J Dermatol Sci 1990;1: 447-453.
144. Minocha Y, Pasricha, J.S, Mohapatra LN, Kandhari, KC. Proteolytic activity of dermatophytes and its role in the pathogenesis of skin lesions. Sabouraudia 1972;10: 79-85.
145. Ragot J, Benedicto T, Taillieu-Calvet G. Comparaison de la kératinolyse in vitro par *Keratinomyces ajelloi, Trichophyton verrucosum, T. gallinae* et *Scopulariopsis brevicaulis*. Bull Soc Franç Mycol Méd 1973;2: 157-161.
146. Calvo MA, Brughera, T, Cabanes, FJ, Calvo, RM, Trape J, Abarca L. Extracellular enzymatic activities of dermatophytes. Mycopathologia 1985;92: 19-22.
147. Rippon JW, Garber ED. Dermatophyte pathogenicity as a function of mating type and associated enzymes. J Invest Dermatol 1969;53: 445-448.
148. Male O. Studium des zytochemischen Verhaltens der Leuzinaminopeptidase sowie einiger Hydrolasen und Enzyme des energieliefernden Stoffwechsels in verschiedenen Wuchsphasen der Pilze. Zbl Bakt I Orig A 1971;217: 345-367.
149. Skorepová M, Hauck H. Extracellular proteinases of *Trichophyton rubrum* and the clinical picture of tinea. Mykosen 1987;30: 25-27.
150. Samdani AJ, Dykes, PJ, Marks R. The proteolytic activity of strains of *Trichophyton mentagrophytes* and *T. rubrum* isolated from tinea pedis and tinea unguium infections. J Med Vet Mycol 1995;33: 167-170.
151. Papini R, Mancianti F. Extracellular enzymatic activity of *Microsporum canis* isolates. Mycopathologia 1996;132: 129-132.
152. Böhme H. Über die Variabilität physiologischer Eigenschaften in der Gattung *Microsporum* Gruby. I. Verwertung von Oleum pedum tauri durch *M. audouinii* und *M. gypseum*. Z Allg Mikrobiol 1972;12: 443-450.
153. Böhme H. Über den Stoffwechsel der Gattung *Microsporum* Gruby. IV. Vergleichende Untersuchungen über den Abbau von Triglyceriden und Wachsen durch fünf Arten der Gattung *Microsporum*. Mycopathologia 1972,46: 221-232.
154. Böhme, H. Unterschiede in der Verwertung von Lipiden durch *Trichophyton rubrum* und *T. mentagrophytes*. Dermatol Monatsschrift 1973;159: 699-704.
155. Hellgren L, Vincent J. Lipolytic activity of some dermatophytes. II. Isolation and characterization of the lipase of *Epidermophyton floccosum*. J Med Microbiol 1981;14: 347-350.
156. Chopra A, Larroya S, Khuller GK. Studies on the phospholipase A of dermatophytes. Curr Microbiol 1981;6: 171-173.
157. Ziegler H, Böhme H. Untersuchungen über den Phosphatstoffwechsel der Dermatomyzeten. Mycopathologia 1966;29: 291-319.
158. Ziegler H, Böhme H. Untersuchungen über den Haarabbau durch Dermatomyzeten. Dermatol Wochenschrift 1963;148: 429-454.
159. Böhme H, Ziegler H. Verbreitung und Keratinophilie von *Anixiopsis stercoraria* (Hansen) Hansen. Arch Klin Exper Dermatol 1965;223: 422-428.
160. Weary PE, Canby CM, Cawley EP. Keratinolytic activity of *Microsporum canis* and *M. gypseum*. J Invest Dermatol 1965;44: 300-310.
161. Böhme H. Über den Befall von Tier- und Menschenhaaren durch Dermatophyten in vitro. Mykosen 1966;9: 63-73.
162. Ragot J. Activité kératinolytique du *Keratinomyces ajelloi* Vanbr. sur la laine non dénaturée. C R Acad Sci (Paris) D 1966;262: 412-415.
163. Ragot J. Influence de certain agents de stérilisation sur la kératine. Mycopathologia 1969;39: 177-186.
164. Weary PE, Canby CM. Keratinolytic activity *of Trichophyton schoenleini, T. rubrum* and *T. mentagrophytes*. J Invest Dermatol 1967;48: 240-248.
165. Kunert J. The digestion of human hair by the dermatophyte *Microsporum gypseum* in a submerged culture. Mykosen 1972;15: 59-71.
166. Kunert J. Keratin decomposition by dermatophytes. II. Presence of S-sulfocysteine and cysteic acid in soluble decomposition products. Z Allg Mikrobiol 1976;16: 97-105.
167. Kunert J. Growth of keratinolytic and non-keratinolytic fungi on human hairs. A physiological study. Acta Univ Olomuc Fac Med 1989;122: 25-38.
168. Hose H, Evans G. Degradation of native keratin by dermatophytes. J Invest Dermatol 1977:68: 245-246.
169. Deshmukh SK, Agrawal SC. In vitro degradation of human hair by some keratinophilic fungi. Mykosen 1982; 25: 454-458.
170. Deshmukh SK, Agrawal SC. Degradation of human hair by some dermatophytes and other keratinophilic fungi. Mykosen 1985;28: 463-466.
171. Safranek WW, Goos RD. Degradation of wool by saprotrophic fungi. Can J Microbiol 1982;28: 137-140.
172. Malviya H, Rajak RC, Hasija SK. Synthesis and regulation of extracellular keratinase in three fungi isolated from the grounds of a gelatin factory, Jabalpur, India. Mycopathologia 1992;120: 1-4.
173. Shrivastava JN, Ghawana VK, Kumar A. Biodegradation of wool by *Trichophyton simii* and *Aspergillus niger*. Mycoses 1996;39: 483-487.
174. Singh CJ, Singh BG, Singh BS. Degradation of certain keratin substrates in vitro by some keratinophilic fungi. Adv Plant Sci India 1995; 8: 271-276.
175. English MP. The saprophytic growth of keratinophilic fungi on keratin. Sabouraudia 1963;2: 115-130.
176. Filipello-Marchisio V, Fusconi A, Rigo S. Keratinolysis and its morphological expression in hair digestion by airborne fungi. Mycopathologia 1994;127: 103-115.
177. McQuade AB. Microbiological degradation of wool. Dermatologica 1964;128: 249-266.
178. Christeller JT. Degradation of wool by *Hofmannophila pseudospretella* larval midgut extracts under conditions simulating the midgut environment. Arch Insect Biochem Physiol 1996;32: 99-119.
179. Böckle B, Müller R. Reduction of disulfide bonds by *Streptomyces pactum* during growth on chicken feathers. Appl Environ Microbiol 1997;63: 790-792.
180. Kamalam A, Thambiah AS. Histological study in Tinea capitis. Mykosen 1981;24: 431-441.
181. Ruffin P, Andrieu S, Biserte G, Biguet J. Sulphitolysis in keratinolysis. Biochemical proof. Sabouraudia 1976;14: 181-184.
182. Kunert J. Biochemical mechanism of keratin degradation by keratinolytic fungi. J Indian Bot Soc 1995;74: 89-98.
183. Dei-Cas E, Vernes A. Parasitic adaptation of pathogenic fungi to mammalian hosts. CRC Crit Rev Microbiol 1986;13: 173-208.
184. Jones HE. Immune response and host resistance of humans to dermatophyte infection. J Am Acad Dermatol 1993;28: S12-S18.
185. Wagner DK, Sohnle PG. Cutaneous defenses against dermatophytes and yeasts. Clin Microbiol Rev 1995;8: 317-335.
186. Weitzman I, Summerbell RC. The dermatophytes. Clin Microbiol Rev 1995;8, 240-259.