

# The rutin catabolic pathway with special emphasis on quercetinase

Sylvain Tranchimand · Pierre Brouant · Gilles Iacazio

Received: 28 December 2009 / Accepted: 8 April 2010 / Published online: 25 April 2010  
© Springer Science+Business Media B.V. 2010

**Abstract** The aim of this review is to give a general account on the oxidative microbial degradation of flavonols. Since now 50 years, various research groups have deciphered the way microorganisms aerobically deal with this important class of flavonoids. Flavonols such as rutin and quercetin are abundantly found in vegetal tissues and exudates, and it was thus patent that various microorganisms will bear the enzymatic machinery necessary to cope with these vegetal secondary metabolites. After initial studies focussed on the general metabolic capacity of various microorganisms towards flavonols, the so called rutin catabolic pathway was rapidly established in moulds. Enzymes of the path as well as substrates and products were known at the beginning of the seventies. Then during 30 years, only sporadic studies were focused on this pathway, before a new burst of interest at the beginning of the new century arose with structural, genomic and theoretical studies mainly conducted towards quercetinase. This is the goal of this work to relate this 50 years journey at the crossroads of microbiology, biochemistry, genetic and chemistry. Some mention of the potential usefulness of the enzymes of the path as well as micro-

organisms bearing the whole rutin catabolic pathway is also discussed.

**Keywords** Rutin catabolic pathway · Quercetinase · Flavonol · Flavonol biodegradation

## Introduction

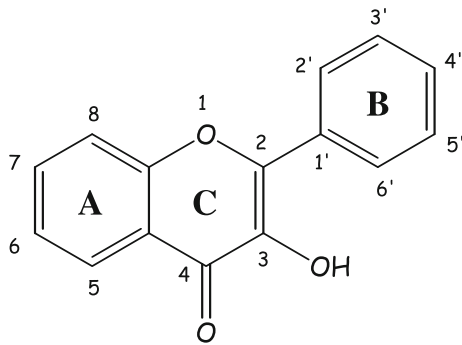
Flavonols are plant secondary metabolites belonging to the wide class of flavonoids (Fig. 1).

In vivo they are produced via the phenyl propanoid pathway. Their structural characteristics are as follow:

- A five carbon atoms skeleton composed of three rings (A, B and C). Ring A and C are fused while ring B (carbon 1') is linked to ring C (carbon 2).
- A double bond between carbon 2 and 3, a hydroxy group on carbon 3 and a keto group on carbon 4.
- Various hydroxy substituents both on ring A and B, the widely encountered substituted positions being C5 and C7 for ring A and C3', C4' and C5' for ring B.
- Various sugars could be found linked to the flavonol aglycone especially in position 3 (as in rutin for example).
- The hydroxy substituents on ring A and C could also be methylated.

The main biological activity associated with flavonols is their antioxidant action arising from their free radical scavenging capacity (Pietta 2000). This

S. Tranchimand · P. Brouant · G. Iacazio (✉)  
BiosCiencs, UMR 6263 ISM2, Université Paul Cézanne  
Aix-Marseille III, Avenue Escadrille Normandie-Niemen,  
13397 Marseille Cedex 20, France  
e-mail: gilles.iacazio@univ-cezanne.fr



**Fig. 1** Flavonol structure and atom numbering

class of flavonoids is especially abundant in fruits and vegetables and as such has an important impact on human nutrition.

Flavonol transformations by microorganisms could be roughly divided into four processes (Das and Rosazza 2006) according to our present knowledge:

#### Microbial bioconversion

##### Anaerobic prokaryotic catabolism

##### Aerobic prokaryotic catabolism

##### Aerobic eukaryotic microbial catabolism (or rutin catabolic pathway).

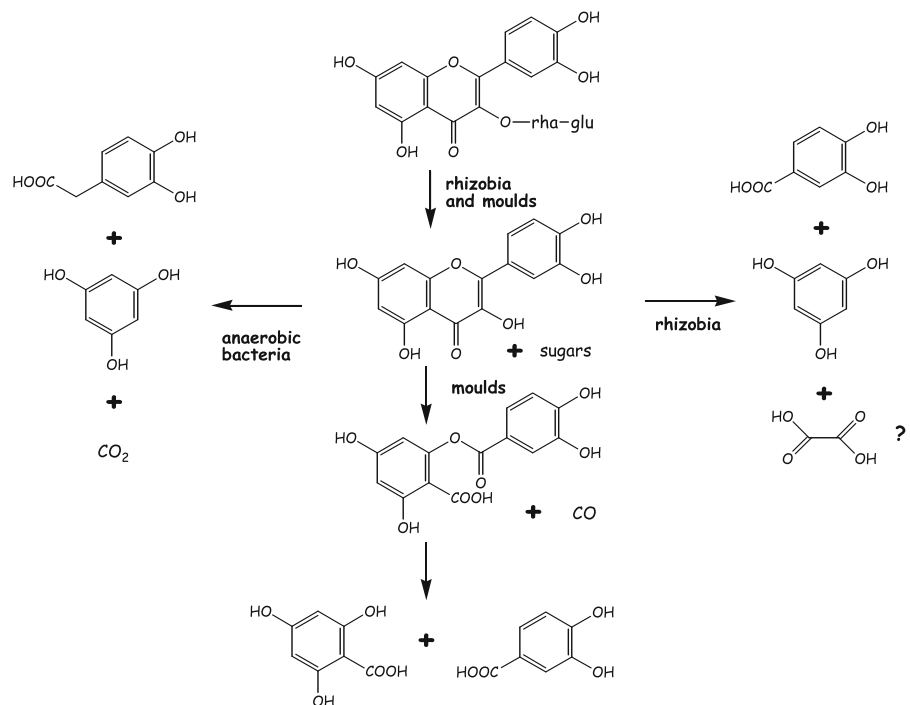
Microbial bioconversion of flavonols has been recently reviewed (Das and Rosazza 2006) and relies either on glycosylation (*Bacillus cereus*), hydroxylation and O-methylation (*Streptomyces griseus*) or O-demethylation (*Aspergillus niger*) of parent compounds.

Flavonol anaerobic prokaryotic catabolism occurs mainly in ruminal and intestinal anaerobic bacteria (Fig. 2). After the removal of the sugar part, if exists, the flavonol aglycone is further transformed in  $\text{CO}_2$ , phloroglucinol and phenyl acetic acid derivatives. Bacteria such as *Eubacterium ramulus* (Schneider and Blaut 2000; Braune et al. 2001), *Clostridium orbiscindens* (Schoefer et al. 2003) and four *Clostridium* sp. (Winter et al. 1989) have been described in this context.

Flavonol aerobic prokaryotic catabolism (Fig. 2) generally takes place in soil bacteria, especially rhizobia (Rao et al. 1991; Rao and Cooper 1994; Pillai and Swarup 2002). Phloroglucinol is one of the formed products together with various benzoic acid derivatives depending on the starting flavonol and possibly oxalic acid for the two remaining carbons.

The aerobic eukaryotic catabolism of flavonols (Fig. 2) is probably the most established of the three

**Fig. 2** Summary of flavonol microbial catabolism (adapted from Das and Rosazza 2006)



catabolic pathways described here. Indeed it is well known that various moulds, especially from the genera *Penicillium* and *Aspergillus*, are able to use rutin as the sole source of carbon and energy during the so-called rutin catabolic pathway. This path involves at least one glycosidase, a dioxygenase (quercetinase) and an esterase. The final products are saccharides (rutinose or the two constituting sugars rhamnose and glucose for rutin), carbon monoxide (evolved during quercetinase catalysis) and two phenolic acids (phloroglucinol carboxylic and protocatechuic acids in the case of rutin).

The aim of this paper is to review the studies on the rutin catabolic pathway in the light of recent work, in order to provide a general account on the subject. A special emphasis will be provided on quercetinase, since this enzyme is the best studied of the path, with an astonishing burst of interest during the last 10 years.

### The rutin catabolic pathway

Metabolites issued from eukaryotic rutin catabolism

The rutin catabolic pathway (RCP) was first established by studying the transformation of rutin either by a resting-cell technique using *Pullularia fermentans* initially grown on rutin (Hattori and Noguchi 1959) or by culture filtrates of *Aspergillus* sp. grown on a rutin based medium (Westlake et al. 1959). In the first study phloroglucinol, protocatechuic acid and the depside between phloroglucinol carboxylic acid and protocatechuic acid were detected. Rutinose (the disaccharide between glucose and rhamnose) and phloroglucinol carboxylic acid were added to this list according to the second study. Taking into account rutinose, phloroglucinol carboxylic acid and protocatechuic acid, 26 over the 27 carbon atoms of rutin are recovered. The last carbon atom, firstly searched as CO<sub>2</sub>, formic acid, formaldehyde or methanol (Westlake et al. 1959) was then soon identified as carbon monoxide (Simpson et al. 1960). Thus at the beginning of the sixties all the products of the path were identified, further suggesting the existence of at least three enzymes: (i) a glycosidase splitting rutin in rutinose and the aglycone quercetin, (ii) an enzyme transforming the latter in carbon monoxide and a

depside, (iii) an esterase acting on the depside and leading to protocatechuic and phloroglucinol carboxylic acids (Fig. 2).

### Microorganisms bearing the RCP

First of all it should be noted that lists of microorganisms able to aerobically degrade rutin or other flavonols are mentioned in the literature (Westlake et al. 1959; Westlake 1963; Westlake and Spencer 1966; Armand-Fraysse and Lebreton 1969; Rao and Weisner 1981; Omori et al. 1986; Narikawa et al. 1998; Rose and Fetzner 2006). In a number of cases the detection of enzyme activities and/or formed products has not been performed. The interested reader is referred to the original papers for an overview of the tested microorganisms. In this chapter we will only focus on strains for which enzymatic activities and/or products of the path have been sufficiently characterised.

Our survey of the literature concerning the detection of either enzymatic activity involved in the RCP or end products of the path for strains grown aerobically in presence of flavonol or glycosylated flavonols is summarised in Table 1.

The RCP has been strictly proved only in moulds. Indeed, only in such microorganisms the three enzymes of the path have been fully characterized. For moulds the enzymatic activities of the RCP are found extracellularly. In this context the genera *Aspergillus* and *Penicillium* are particularly over-represented (see Table 1).

For yeasts or yeast like fungi the RCP could be judged as probable since the three end products of the path, namely carbon monoxide, phloroglucinol carboxylic acid and protocatechuic acid (Westlake and Spencer 1966) have been detected, as well as the intermediate depside (Hattori and Noguchi 1959) from experiments using rutin, quercitrin or quercetin as substrates. When search for, the three enzymatic activities of the path have been proven to be intracellular (Westlake and Spencer 1966).

The fate of bacteria in relation with the RCP is less secure. From recent studies the presence of a quercetinase in bacteria such as *Bacillus subtilis* (Bowater et al. 2004) or a *Streptomyces* sp. (Merkens et al. 2007) is now evidence. But for the two bacteria the presence of a quercetinase looks to be related much more to a detoxification process rather than a catabolic one. To the best of our knowledge, the two other enzymes

**Table 1** List of micro-organisms bearing one or more enzyme of the RCP or leading to one or more of the end products of the RCP when grown aerobically in the presence of flavonol

Microorganisms (taxonomic status)	Enzymes			Products			References
	R	Q	E	D	CO	PGCA	
<i>Alternaria</i> sp. (M)				✓	✓		Westlake et al. (1961)
<i>Aspergillus flavus</i> (M)	✓	✓	✓	✓	✓	✓	Westlake et al. (1959, 1961), Hay et al. (1961), Westlake and Simpson (1961), Child et al. (1963)
<i>Aspergillus niger</i> (M)	✓	✓		✓	✓		Westlake et al. (1959), Simpson et al. (1960), Haluk and Metche (1970), Hund et al. (1999)
<i>Aspergillus japonicus</i> (M)		✓	✓				Fusetti et al. (2002)
<i>Cephalosporium</i> sp. (M)				✓	✓		Westlake et al. (1961)
<i>Diaporthe</i> sp. (M)				✓			Westlake et al. (1961)
<i>Fusarium oxysporum</i> (M)				✓	✓		Barz (1971)
<i>Penicillium brevicompactum</i> (M)	✓						Narikawa et al. (1998)
<i>Penicillium decumbens</i> (M)		✓					Mamma et al. (2004)
<i>Penicillium funiculosum</i> (M)	✓						Narikawa et al. (1998)
<i>Penicillium herquei</i> (M)	✓						Narikawa et al. (1998)
<i>Penicillium olsonii</i> (M)		✓		✓	✓		Iacazio (2005), Tranchimand et al. (2008)
<i>Penicillium rugulosum</i> (M)	✓						Narikawa et al. (1998)
<i>Pullularia fermentans</i> (YLF)				✓	✓		Hattori and Noguchi (1959), Noguchi (1963)
<i>Pullularia pullulans</i> (YLF)	✓	✓	✓	✓	✓		Westlake and Spencer (1966)
<i>Cryptococcus albidus</i> (Y)	✓	✓	✓	✓	✓		Westlake and Spencer (1966)
<i>Cryptococcus diffluens</i> (Y)	✓	✓	✓	✓	✓		Westlake and Spencer (1966)
<i>Bacillus subtilis</i> (B)		✓					Bowater et al. (2004)
<i>Corynebacteria</i> sp. (B)					✓		Omori et al. (1986)
<i>Streptomyces</i> sp. (B)		✓					Merkens et al. (2007)

M mould; YLF yeast-like fungi; Y yeast; B bacteria; R rutinase; Q quercetinase; E esterase; D depside; CO carbon monoxide; PGCA phloroglucinol carboxylic acid

of the path have not been reported to date in prokaryotes.

### Enzymes involved in the RCP

Two of the three types of enzyme involved in the RCP (namely glycosidase and esterase) will be discussed in some details in this chapter and the last one, quercetinase, very briefly since it is the main subject of the following parts of this review.

### Glycosidases

As for rutin, flavonols can bear various sugars especially those linked to position 3 (glu-rha or

rutinose in the case of rutin). As quercetinases are not able to transform such 3-glycosylated flavonols, the microorganism should rely on a glycosidase activity to release sugars and the flavonol aglycone to allow complete catabolism. Depending on the glycosylated flavonol used as substrate by the microorganism, more or less specific glycosidases have been shown to be produced.

*Rutin(oxid)ase* The first glycosidase to be described in the context of the RCP was called rutinase [from *Aspergillus flavus* (Hay et al. 1961)] and later on rutinoidase [from *Penicillium rugulosum* (Narikawa et al. 2000)] and shown to hydrolyse rutin in quercetin and rutinose (Fig. 3). These two enzymes were produced extracellularly.

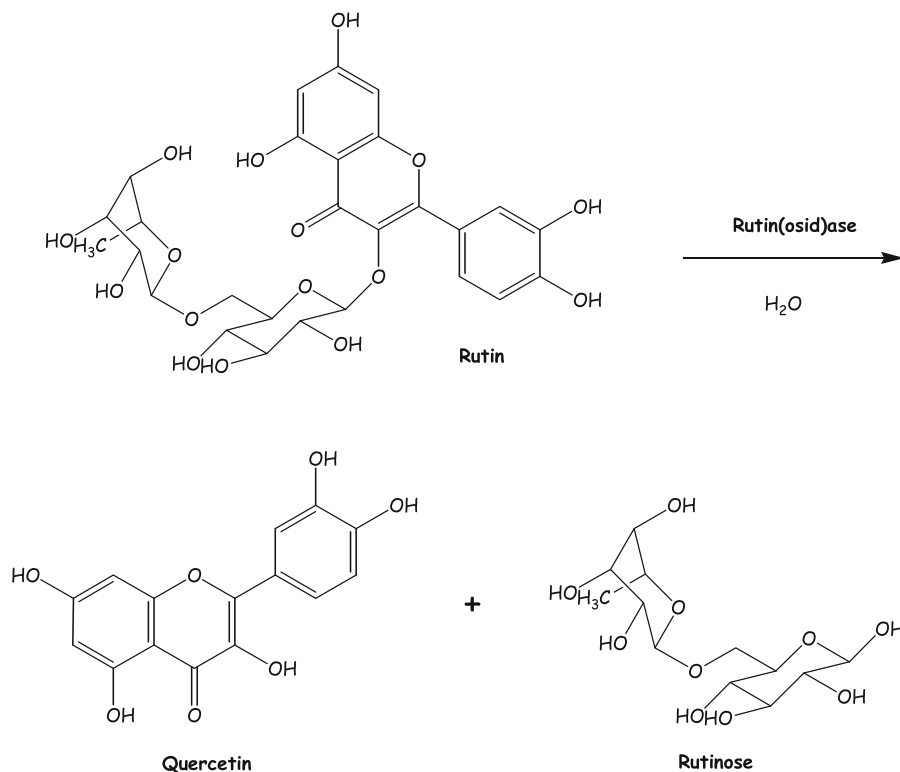
To the best of our knowledge the papers of Westlake et al. (Westlake et al. 1959; Hay et al. 1961; Westlake and Simpson 1961) and Narikawa et al. (1998, 2000) are the only ones describing an enzyme (purified or not) leading to rutinose plus quercetin upon action on rutin.

The *Aspergillus* enzyme (Hay et al. 1961) was shown to be induced strongly by rutin and hyperosid (quercetin-3-*O*-galactose), well induced by three non-glycosylated flavonols (rhamnetin, kaempferol and quercetin), poorly induced by two phenolic acids (2,4-dihydroxy benzoic and 3,4-dihydroxyphenyl acetic acids) and not induced by glucose, galactose and rutinose. The purified enzyme was shown to be relatively stable, having an optimum pH of 5.6 and being inhibited by organic bases such as pyridine and dimethyl amine (92 and 84% inhibition respectively at 0.01 and 0.02 M). The substrate specificity of the enzyme was shown to be quite restricted. Indeed within glycosylated flavonols only rutin and hyperosid were hydrolysed while quercitrin (quercetin 3-*O*- $\alpha$ -rhamnoside) was not as well as a number of small methyl glycoside (from the following sugars:  $\beta$ -arabinose,  $\alpha$ -glucose,  $\beta$ -glucose,  $\beta$ -maltose,  $\alpha$ -mannose and

$\alpha$ -rhamnose). The  $K_m$  versus rutin was found to be  $7.3 \times 10^{-5}$  M. During rutin hydrolysis a 1–1 ratio was found between the two products (quercetin and rutinose).

The *Penicillium* enzyme (Narikawa et al. 2000) was purified to apparent homogeneity as an homotetramer (245 kDa for the native enzyme, 65 kDa for the monomer) with a pI of 5, an optimum pH around 2 and an optimal temperature of 50°C, the enzyme being stable below 40°C. The enzyme, obtained from *Penicillium rugulosum* grown on rutin, has a rather narrow substrate specificity since only rutin and isoquercitrin (quercetin 3-*O*- $\beta$ -glucoside) were substrates while quercitrin (quercetin 3-*O*- $\alpha$ -rhamnoside), hesperidin (hesperitin 7-*O*- $\beta$ -rutinoside), naringin (naringenin 7-*O*- $\beta$ -neohesperidoside) were not substrate at all as well as 4-nitrophenyl  $\alpha$ -rhamnoside, 4-nitrophenyl  $\beta$ -glucoside, methyl  $\beta$ -glucoside and cellobiose. From the two studies mentioned above it is noteworthy that rutin(osid)ase is a quite specific enzyme discriminating both the sugar part and the flavonol aglycone part of its substrate and is clearly not a simple  $\beta$ -glucosidase or a simple rutinosidase (in the meaning of an enzyme acting on rutinoside conjugates).

**Fig. 3** Reaction catalysed by rutin(osid)ase



***β-Glucosidase*** Within micro-organisms bearing the RCP, some studies have been devoted to the search of a *β*-glucosidase activity (Mamma et al. 2004; Medina et al. 2004, 2005). This activity could be used by these microorganisms to cleave flavonol glycosides such as isoquercitrin (quercetin 3-*O*-*β*-glucoside).

In a study devoted to the production of extracellular enzymes obtained from *Penicillium decumbens* when grown on rutin (Mamma et al. 2004), three activities were search for (*β*-glucosidase, *α*-rhamnosidase and quercetinase). The *β*-glucosidase was quantified using *p*-nitrophenyl-*β*-*D*-glucopyranoside but no analysis of the action of the purified enzyme on rutin or rutinose was reported. The enzyme was analysed as a tetramer of 480 kDa with an optimum pH of 7 and an optimal temperature of 60°C.

Using the same *Aspergillus flavus* strain used by Westlake et al. nearly 40 years ago, a proteomic analysis of rutin-induced secreted proteins was conducted and compared to a non-rutin containing potato dextrose medium (Medina et al. 2004, 2005). A *β*-glucosidase activity was found to be induced in the rutin medium, although the same activity was detected in the potato dextrose medium but in a lower amount. The substrate specificity of the enzyme was not investigated, the *β*-glucosidase activity being detected using *p*-nitrophenyl-*β*-*D*-glucopyranoside. In the context of RCP *α*-*L*-rhamnosidase and quercetinase activities were also assessed.

From these three studies the production of extracellular *β*-glucosidase activity have been proved in moulds when grown on rutin based medium but its relation with potential substrates of the path (rutin or isoquercitrin for example) have not yet been tested.

***α-Rhamnosidase*** Within the RCP the enzyme *α*-rhamnosidase could be involved either in the cleavage of rutinose in rhamnose plus glucose or in the cleavage of rutin in rhamnose plus isoquercitrin (Fig. 4), the latter case being the best documented.

Numerous studies have demonstrated that various moulds when grown on or induced by *L*-rhamnose or flavonoid glycosides containing *L*-rhamnose, produce an extracellular *α*-*L*-rhamnosidase activity which in some cases has been proved to cleave rutin (Kurosawa et al. 1973; Manzanares et al. 1997, 2000, 2001; Gallego et al. 2001; Monti et al. 2004; Puti and Kalra 2005). These activities classified as hesperidinase,

naringinase or simply *α*-*L*-rhamnosidase are more or less specific. The reported studies have not been conducted in relation with the RCP, but it should be noted that filamentous fungi of the genera *Aspergillus* are over represented. In Table 2 are grouped some of the moulds producing an *α*-*L*-rhamnosidase showing activity on rutin.

Some of the properties of various *α*-*L*-rhamnosidas-es proved to be able to act on rutin are grouped in Table 3.

***Quercitrinase*** This enzyme was detected (Westlake 1963) in a survey of moulds able to grow on and to metabolise quercitrin (quercetin 3-*L*-rhamnoside). It catalyses the cleavage of quercitrin into *L*-rhamnose and quercetin (Fig. 5).

In this context two strains of *A. flavus* were found to produce an extracellular quercitrinase activity, the released quercetin being transformed further in PGCA, PA and CO by these strains. The enzyme, with an optimum pH of 6.4, was highly induced by flavonol rhamnosides (myricitrin, quercitrin, robinin) and well induced by rutinose and galactoside flavonols (rutin, hyperosid) as well as flavonols (kaempferol, quercetin, rhamnetin, morin, myricetin) but not by *L*-rhamnose. Rutinase and quercetinase activities were also detected using the same inducers, further suggesting that these three extracellular activities were induced by the flavonol skeleton or a product of its degradation.

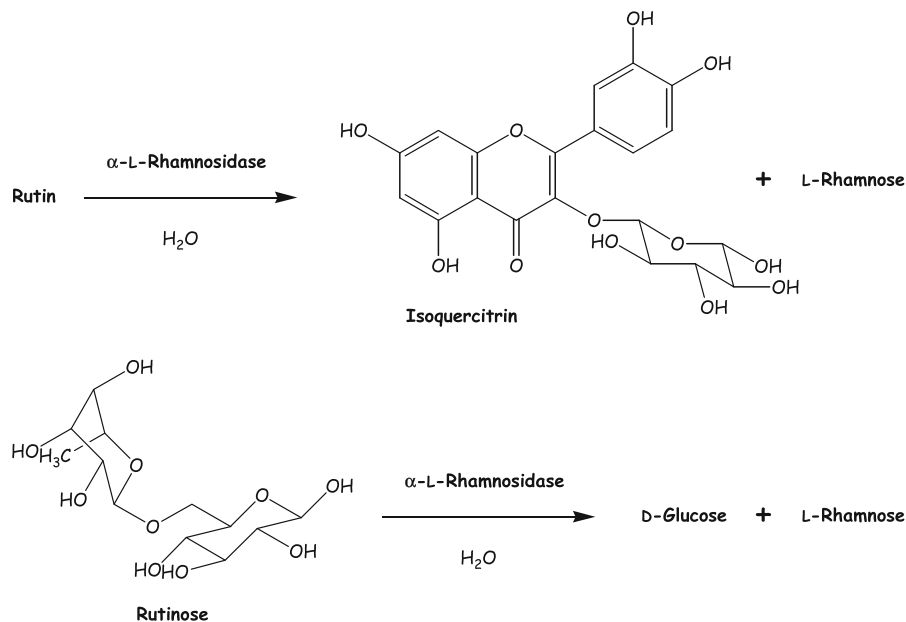
Although the enzyme could be viewed as an *α*-rhamnosidase, its substrate specificity is restricted to flavonol rhamnosides (myricitrin, quercitrin, robinin). Indeed numerous *L*-rhamnose containing compounds were not hydrolysed (rutin, hesperidin, naringin, methyl rhamnoside), thus distinguishing quercitrinase from the *α*-*L*-rhamnosidas-es discussed above.

#### *Quercetinase*

For flavonol glyco-conjugates and following the action of glycosidase(s), the flavonol aglycones are released from the corresponding glycosides and further processed through quercetinase action in the RCP (Fig. 6).

The products of the reaction are carbon monoxide and a depside. Detailed analysis of quercetinases will be the subject of a following chapter.

**Fig. 4** Possible involvement of  $\alpha$ -L-rhamnosidase in the context of the RCP



### Esterase

The last enzyme of the RCP is an esterase hydrolysing the formed depside following quercetinase action, into the two corresponding phenolic acids (Fig. 7). The latter could then enter general catabolic pathway such as the Krebs cycle after ring cleavage and adequate transformation in an intermediate of this path.

To the best of our knowledge only the enzyme from *A. flavus* has been characterised to date (Child et al. 1963, 1971). The esterase is produced extracellularly on a rutin based medium and could alternatively be induced by addition of flavonols (glycosylated or not) on a glucose based medium, the depside arising from quercetin by the action of quercetinase and tannic acid (Child et al. 1963). This esterase was purified and characterised (Child et al. 1971). The enzyme has an optimal pH of 4.3, a pI of 4.45 and is a glycoprotein of 166 kDa bearing 42.8% of sugars. The substrate specificity of the enzyme was also studied. The enzyme acts on depsides derived from flavonols metabolised through the RCP, tannic acid but not on simpler aromatic esters such as methyl esters of phenolic acids or acetates of various phenols (Child et al. 1963). A further in depth study (Child et al. 1971) of depsides susceptible to be hydrolysed by the esterase has revealed that the phenolic having its carboxylic acid function involved in the ester linkage of the depside

should bear at least one and up to three hydroxy functionalities (but not in ortho from the ester linkage) and can't bear a methoxy or a nitro group. This part of the depside is structurally the most important since the alcohol part could be either a phenolic or an aliphatic alcohol (methanol). This esterase looked to be thus rather specific of esters of *p*-hydroxy benzoic, protocatechuic, 3,5-dihydroxy benzoic and gallic acids.

### Quercetinases from moulds

#### Quercetinase production and induction

##### Production

The extracellular production of quercetinase is actually only known from moulds. It is generally done on a mineral liquid medium supplemented with rutin as the sole or main carbon and energy source [*A. flavus* (Westlake et al. 1959; Westlake and Simpson 1961; Simpson et al. 1963; Medina et al. 2004, 2005), *A. niger* (Hund et al. 1999), *P. decumbens* (Mamma et al. 2004), *P. olsonii* (Iacazio 2005)] or on quercetin [*Aspergillus* sp. (Padrn et al. 1960), *A. japonicus* (Kooter et al. 2002)].

Some experiments have been dedicated to quercetinase production improvement through medium engineering. The group of Westlake (Padrn et al. 1960;

**Table 2** Fungal strains producing an extracellular  $\alpha$ -L-rhamnosidase active against rutin

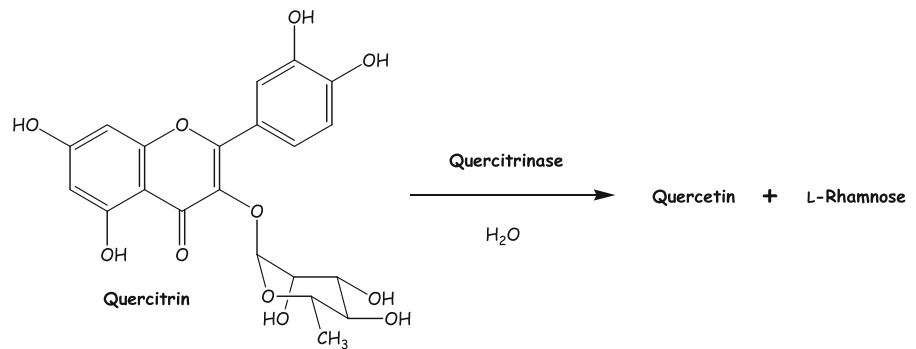
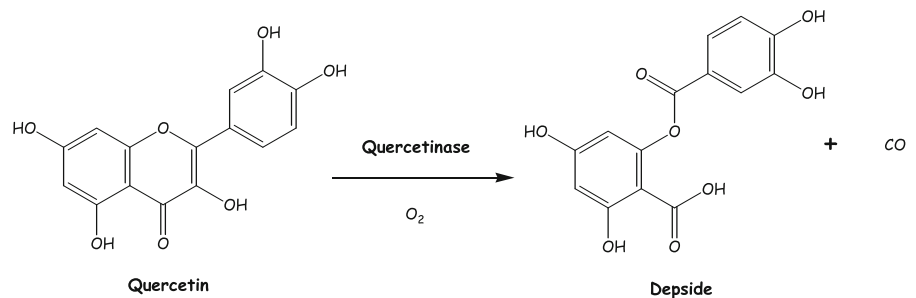
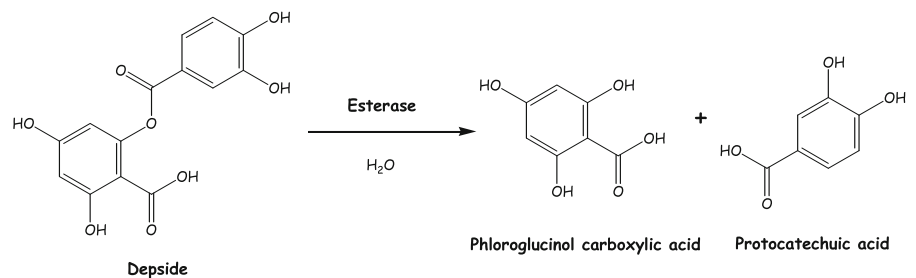
Strains	References	Carbon source or inducer	Relative activity (%)
<i>Aspergillus nidulans</i>	Manzanares et al. (2000)	L-Rhamnose	Naringin: 100 Rutin: 42 Hesperidin: 5
<i>Emericella nidulans</i>	Monti et al. (2004)	L-Rhamnose, hesperidin, naringin	Not reported, quercitrin acting as a substrate
<i>Aspergillus aculeatus</i>	Manzanares et al. (2001)	Hesperidin	Rhamnosidase A B Naringin: 100 49 Rutin: 92 100 Hesperidin: 4 6 Quercitrin: 0 0
<i>Aspergillus terreus</i>	Gallego et al. (2001)	L-Rhamnose	Not reported
<i>Aspergillus terreus</i>	Monti et al. (2004)	L-Rhamnose, rutin, hesperidin, naringin	Not reported
<i>Aspergillus niger</i> (hesperidinase commercial preparation)	Manzanares et al. (1997)	Unknown	Naringin: 40 Rutin: 100 Hesperidin: 29 Quercitrin: 0
<i>Aspergillus niger</i>	(Puti and Kalra 2005)	Naringin	Naringin: 100 Rutin: 63 Hesperidin: 0
<i>Aspergillus niger</i>	Monti et al. (2004)	Rutin, hesperidin, naringin	Not reported
<i>Aspergillus aculeatus</i>	Monti et al. (2004)	L-Rhamnose, rutin, hesperidin, naringin	Not reported
<i>Fusarium oxysporum</i>	Monti et al. (2004)	Rutin, naringin	Not reported
<i>Mortierella alpina</i>	Monti et al. (2004)	Rutin	Not reported, quercitrin acting as a substrate
<i>Talaromyces flavus</i>	Monti et al. (2004)	L-Rhamnose, naringin	Not reported, quercitrin acting as a substrate

**Table 3** Characteristics of some extracellular mould  $\alpha$ -L-rhamnosidases

Strain (reference)	M.W. (kDa)	pI	pH <sub>opt.</sub>	T <sub>opt.</sub> (°C)	K <sub>m</sub> (mM)	V <sub>max</sub> (U/mg)	K <sub>i</sub> (mM)
<i>A. terreus</i> (Gallego et al. 2001)	96	4.6	4	44	0.17 <sup>b</sup>	84	2.5 <sup>d</sup>
<i>A. nidulans</i> (Manzanares et al. 2000)	102	5	4.5–6	60	0.27 <sup>b</sup>	64.6	0.3 <sup>d</sup>
<i>A. acculatus</i> RhaA (Manzanares et al. 2001)	92	6.2	4.5–5		2.8 <sup>b</sup>	24	4.2 <sup>d</sup>
<i>A. acculatus</i> RhaB (Manzanares et al. 2001)	85	5.2–5.9	4.5–5		0.3 <sup>b</sup>	14	1.5 <sup>d</sup>
<i>A. niger</i> (Puti and Kalra 2005)	168 <sup>a</sup>		4	50	1.9 <sup>c</sup>	21	
<i>A. niger</i> (Manzanares et al. 1997)	85	4.5–5.2	4.5	65	2.9 <sup>b</sup>	20.6	3.5 <sup>d</sup>
<i>A. niger</i> (Kurosawa et al. 1973)			4.5		2.65 <sup>b</sup>		

<sup>a</sup> Monomer 85 kDa<sup>b</sup> *p*-Nitrophenyl- $\alpha$ -L-rhamnopyranoside<sup>c</sup> Naringin<sup>d</sup> L-Rhamnose



**Fig. 5** Reaction catalysed by quercitrinase**Fig. 6** Reaction catalysed by quercetinase**Fig. 7** Reaction catalysed by esterase

Simpson et al. 1963) has shown that rutin and copper salt concentrations,  $(NH_4)_2SO_4$  as well as the nitrogen source and the presence of  $MgCl_2$  influenced markedly the extracellular production of quercetinase by *A. flavus*. A maximum of 0.63 U/ml (one unit is defined as the amount of enzyme oxidizing 1  $\mu$ mol of quercetin per minute) was reported (Simpson et al. 1963). With *P. decumbens* (Mamma et al. 2004) initial culture pH (pH 7), rutin concentration (8 g/l), and nitrogen source and concentration ( $(NH_4)_2SO_4$ , 9 g/l) were studied and optimized, leading to an increased quercetinase production up to 0.64 U/ml. In a work specifically dedicated to quercetinase production improvement using fractional factorial design methodology (Iacazio 2005), quercetinase production by *P. olsonii* was improved sixfold (from 0.12 to 0.71 U/ml). Also some parameters were shown to be important, the

heaviest one was found to be the concentration of yeast extract (3.2 g/l).

### Induction

By growing *A. flavus* on a glucose based liquid medium (Simpson et al. 1963) it has been shown that quercetinase is strongly induced by kaempferol, rutin, hyperosid and quercetin, poorly induced by quercitrin and fisetin and not induced by flavonol (3-hydroxy flavone) and robinetin, all these compounds being flavonols. Within the same study various flavones, flavans-3-ol, flavanones and one chalcone were found not to be inducers of quercetinase activity as well as various simple aromatics (phloroglucinol and protocatechuic acid) and sugars like glucose, rhamnose and rutinose. Recently (Tranchimand et al. 2005), a

study devoted to the induction of quercetinase activity in *P. olsonii* grown on a glucose based medium, established that it is probably the final phenolic acid arising from ring A after flavonol cleavage (PGCA in the case of quercetin) that is the true inducer. Indeed rutin, quercetin, the depside between PGCA and PA and PGCA itself were proved to be inducers. To the contrary glucose, rhamnose and PA were not inducers nor was phloroglucinol. The necessity of three hydroxy functionalities on the benzoic acid skeleton (on position 2, 4 and 6 like in PGCA) was also investigated. Dihydroxy derivatives (2,4 and 2,6-dihydroxy benzoic acid) were still inducers but not monohydroxy derivatives (2- and 4-hydroxy benzoic acid). In the context of the RCP it is probably PGCA which acts as inducer of quercetinase activity when 5,7 dihydroxy substituted flavonols are available, at least for *P. olsonii*.

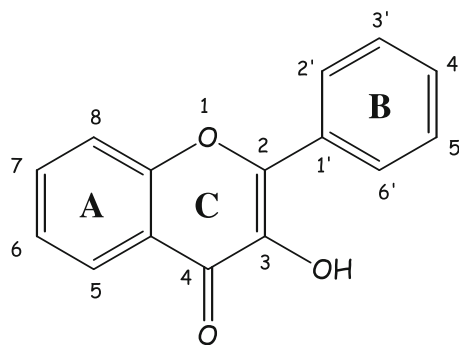
#### Substrate specificity

Quercetinases are actually considered to act only on free flavonols. Indeed related compounds such as glycosylated flavonols (Hay et al. 1961), flavonoids other than flavonols (Padrn et al. 1960; Simpson et al. 1963; Oka et al. 1972), as well as 3,5,7-trihydroxy chromone (Simpson et al. 1963) or 1*H*-3-hydroxy-4-oxoquinoline and 1*H*-3-hydroxy-4-oxoquinaldine (Hund et al. 1999) are not substrates. The minimal substrate structure is a 3-hydroxy flavone skeleton (Fig. 8) bearing various substitutions (essentially hydroxy group) on ring A (main positions 5 and 7) and B (main positions 3', 4' and 5').

The necessity of both a C2–C3 double bond and a free 3-hydroxy substitution (and thus of a 4-oxo group to stabilised this enolic form) is well documented (Padrn et al. 1960; Simpson et al. 1963; Oka et al. 1972). It should be noted that the B ring could be replaced by a methyl group, although this compound (2-methyl-3,5,7-trihydroxychromone) is a poor substrate (Simpson et al. 1963).

The substitution pattern on ring A and B influences both the rate of flavonol degradation and the  $K_m$  of the enzyme. Table 4 resumes a study conducted with a purified quercetinase from *A. flavus* (Oka et al. 1972).

The highest degradation rate is found for flavonols possessing both 5- and 7-hydroxy substitutions whatever the substitution pattern on the B ring (except



**Fig. 8** The minimal 3-hydroxy flavone skeleton required for quercetinase action

for morin, 2'-OH substitution and fisetin only 7-OH substitution). In this series the highest rate of degradation is found for kaempferol bearing only one hydroxy substitution on ring B in position 4'. The lowest  $K_m$  (in the micromolar range) are found for flavonols bearing one or two hydroxy substitutions on ring A (7-OH and 5,7-diOH) and none, one or two hydroxy substitutions on ring B (4'-OH and 3',4'-diOH). Similar conclusions could be drawn from a recent study on purified *P. olsonii* quercetinase (Tranchimand et al. 2008). In the latter the nitrogen equivalent of flavonol (i.e. 1*H*-2-benzyl-3-hydroxy-4-oxoquinoline) was found not to be a substrate.

#### Products of the reaction

The formation of the depside during the degradation of rutin by resting cells of *Pullularia fermentens* was recognised very early (Hattori and Noguchi 1959). Its structure was assigned through identification of its hydrolytic products PGCA and PA, and from the structure of the starting compound rutin. The same metabolite was soon after found from quercetin incubation with three different rutin grown *A. flavus* filtrates (Westlake et al. 1959). Later on spectroscopic data confirmed the assigned structure for the depside obtained from quercetin (Omori et al. 1986; Child et al. 1971; Hund et al. 1999; Krishnamachari et al. 2002; Bowater et al. 2004; Barney et al. 2004; Tranchimand et al. 2006) as well as from kaempferol and myricetin (Tranchimand et al. 2006). As the degradation of rutin generates rutinose, PGCA and PA that account for 26 of the 27 carbons initially present, the remaining carbon (C3–OH) was first

**Table 4** Relative rate of degradation (quercetin 100%) and  $K_m$  of various flavonol substrates according to their substitution pattern using purified *A. flavus* quercetinase

Substrate (trivial name)	Substitution pattern (OH)							$K_m$	Rel. rate (%)
	5	7	8	2'	3'	4'	5'		
								$3.5 \times 10^{-5}$ M	0.05
						✓		$1.3 \times 10^{-5}$ M	2.02
		✓						$7 \times 10^{-6}$ M	0.9
		✓			✓	✓		$4 \times 10^{-5}$ M	2.01
Fisetin		✓				✓		$4.1 \times 10^{-6}$ M	23.9
		✓				✓		$4.2 \times 10^{-5}$ M	58.0
Robinetin		✓	✓		✓	✓		$2.3 \times 10^{-4}$ M	11.2
		✓	✓		✓	✓	✓	$1 \times 10^{-4}$ M	6.97
	✓ <sup>a</sup>	✓			✓	✓		$1 \times 10^{-4}$ M	7.3
Galangin	✓	✓						$4 \times 10^{-5}$ M	29.7
Kaempferol	✓	✓				✓		$8 \times 10^{-6}$ M	254
Quercetin	✓	✓			✓	✓		$5.2 \times 10^{-6}$ M	100
Morin	✓	✓		✓		✓		$4.6 \times 10^{-6}$ M	0.216
Myricetin	✓	✓			✓	✓	✓	$2.1 \times 10^{-5}$ M	85.7

<sup>a</sup> OMe substitution. Adapted from Oka et al. (1972)

search unsuccessfully as CO<sub>2</sub>, methanol, methanal or formic acid (Westlake et al. 1959). By using rutin specifically labelled on carbon 3 (<sup>14</sup>C–OH) among other techniques, the missing carbon was found to evolve as carbon monoxide (Simpson et al. 1960). The enzyme responsible for the production of both the depside and CO was characterised from *A. flavus* and named quercetinase (Simpson et al. 1960).

#### Purification and characterisation of the enzyme

Up to date the purification and characterisation of five mould quercetinases [*A. flavus* (Simpson et al. 1963; Oka et al. 1971, 1972; Oka and Simpson 1972), *A. niger* (Hund et al. 1999), *P. decumbens* (Mamma et al. 2004), *A. japonicus* (Fusetti et al. 2002; Kooter et al. 2002) and *P. olsonii* (Tranchimand et al. 2008)] have been reported. The enzyme is easily purified by classical chromatographic procedures (ion exchange chromatography and gel filtration). Published characteristics of purified quercetinases are summarised in Table 5.

From a general point of view mould extracellular quercetinases are acidic glycoproteins, active at acidic pHs. It should be noted that enzymatic deglycosylation of recombinant *A. japonicus* quercetinase has no effect

on its catalytic efficiency (Steiner et al. 2002a). Mould quercetinases have been purified as monomer, homodimer or homotetramer except the one from *A. niger* which was reported as a heterotrimer (Hund et al. 1999). Mould quercetinases are quite robust as could be expected for extracellular enzymes, the one from *A. flavus* (Simpson et al. 1963) being stable for one month at 0 and –20°C and 10 months when stored lyophilised.

#### Metal content

In 1971, it was reported for the first time that *A. flavus* quercetinase was a metalloprotein (Oka and Simpson 1971). By atomic absorption spectrometry the metal was found to be copper at an approximate concentration of 2 eq. per enzyme molecule, and not iron. This result was strengthened by inhibition studies using chelators specific for copper ions (ethylxanthate, diphenylthiocarbazon, toluene-3,4-dithiol and diethyldithiocarbamate), which were found to be strong inhibitors and a chelator specific for iron (1,2-dihydroxy-benzene-3,5-disulfonate) which was found to be a poor inhibitor. Further complexation studies with 2,2'-bisquinoline were indicative that copper was in the cuprous state and that this state was

**Table 5** Physico-chemical characteristics of various purified quercetinases

	<i>A. flavus</i> 1 <sup>a</sup>	<i>A. flavus</i> 2 <sup>a</sup>	<i>A. niger</i>	<i>P. decumbens</i>	<i>A. japonicus</i> 1 <sup>b</sup>	<i>A. japonicus</i> 2 <sup>b</sup>	<i>P. olsonii</i>	
Molecular weight (kDa)	130–140 <sup>c</sup> 111 <sup>d</sup>	80	130–170 <sup>e</sup>	135 <sup>c</sup>	180 <sup>e,f</sup>	45 <sup>e,g</sup>	50	55
Specific activity <sup>h</sup>	180 U/mg	173 U/mg	55.7 U/mg	45.3 U/mg	177 U/mg	90 U/mg	175 U/mg	
K <sub>m</sub> (quercetin)	5.2 μM		6.6 μM				1.1 μM <sup>i</sup>	19 μM <sup>j</sup>
Optimal pH	5.5–6		5.5–6.7 <sup>k</sup> 6.0 <sup>l</sup>	2.0–6			6.5	
Optimal temperature			40°C	50°C			40°C	
Sugar content	27.5%	4.7%	46–54%		25%		16% at least	
pI	4.12	4.05					5.55, 5.81, 6.07, 6.38 <sup>m</sup>	6.00 <sup>n</sup>
Quaternary structure	Monomeric	Monomeric	Heterotrimeric	Homotetrameric	Homodimeric	Homodimeric	Monomeric	Monomeric

<sup>a</sup> From *A. flavus* two quercetinases varying in their sugar content have been purified and were thought to differ only by this character

<sup>b</sup> For *A. japonicus* both native (1) and recombinant [expressed in *A. awamori* (2)] quercetinases were purified and partially characterised

<sup>c</sup> By gel filtration

<sup>d</sup> By sedimentation

<sup>e</sup> By SDS–PAGE

<sup>f</sup> For the homotetramer

<sup>g</sup> For the monomer

<sup>h</sup> 1 U = 1 μmol of degraded quercetin per minute

<sup>i</sup> With 1% DMSO

<sup>j</sup> With 20% DMSO

<sup>k</sup> With MES buffer

<sup>l</sup> With Britton–Robinson buffer

<sup>m</sup> Native form

<sup>n</sup> Deglycosylated form

the catalytically active one. Indeed reduction of the enzyme (or more exactly of copper to the cupric state) was strongly inhibitory. Confirmation of the nature of the metallic cofactor came later with a work dedicated to the purification and characterisation of *A. niger* quercetinase (Hund et al. 1999). By atomic absorption spectrometry the copper content of the enzyme was found to be 1–1.6 eq. In the same work the presence of a non-blue type 2 cuprous ion was revealed by EPR spectroscopy. A precise copper content in a quercetinase (from *A. japonicus*, heterologously expressed in *A. awamori*) came from studies devoted to the structural characterisation of this enzyme by X-ray crystallography. The enzyme was proved to have one copper atom per molecule of protein (Fusetto et al. 2002; Steiner et al. 2002a, b, c) in good accordance

with a 0.8 eq. of copper found by atomic absorption spectroscopy (Kooter et al. 2002). Recently, using the same technique, a copper content of  $0.9 \pm 0.1$  atom per monomer was found for *P. olsonii* quercetinase (Tranchimand et al. 2008). Thus actually mould quercetinases are thought to all have one cuprous ion per molecule as cofactor.

#### Quercetinase inhibition

Inhibitors of quercetinase could be roughly divided into three groups (i) compounds acting as copper chelator (ii) those acting as copper reductant and (iii) those structurally related to quercetinase substrates.

Specific copper chelates such as ethylxanthate, diphenyldithiocarbazon, diethyldithiocarbamate (DDC) and

toluene-3,4-dithiol are strong inhibitors of *A. flavus* quercetinase, acting in the micromolar range (Oka et al. 1971, 1972). The inhibition caused by ethylxanthate was found to be competitive ( $K_i = 2.7 \times 10^{-7}$  M). Using purified *A. niger* quercetinase similar results were obtained with the first three inhibitors listed above (Hund et al. 1999) and DDC inhibited totally *P. olsonii* quercetinase at 100 nM concentration (Tranchimand et al. 2008). With *A. flavus* quercetinase, nitrogen bases with chelating capacities such as 8-hydroxy quinoline, *o*-phenanthroline and  $\alpha, \alpha'$ -dipyridyl were found more potent inhibitors than non-chelating bases such as  $\alpha$ -naphthoquinoline, quinoline and pyridine (Oka et al. 1972).

The second class of quercetinase inhibitors is sulfhydryl reducing agents such as sodium dithionite, mercaptoethanol and dithiothreitol. Their action relies on copper ion reduction from  $\text{Cu}^{2+}$  to  $\text{Cu}^+$ . Indeed the inhibition of *A. flavus* quercetinase has been proved to be reversible and activity recovery was enhanced by shaking in air (Oka et al. 1972).

Up to now inhibitors structurally related to quercetinase substrates have only been found in the flavonol family. Indeed two very poor quercetinase substrates, flavonol and morin, were identified as potent competitive inhibitors of *A. flavus* quercetinase with a respective  $K_i$  of 7.4 and 6  $\mu\text{M}$  (Simpson et al. 1963; Oka et al. 1972). Interestingly, although flavonol was not an inhibitor of *P. olsonii* quercetinase nor a substrate, its nitrogen equivalent (i.e. 1*H*-2-benzyl-3-hydroxy-4-oxoquinoline) was found to be a competitive inhibitor with a  $K_i$  of 4  $\mu\text{M}$  but not a substrate (Tranchimand et al. 2008). Two flavones and a dihydroflavonol (taxifolin) were not inhibitors of *A. flavus* quercetinase (Oka et al. 1972) nor was taxifolin an inhibitor of *P. olsonii* quercetinase (Tranchimand et al. 2008). All these results suggest that to act as structurally related quercetinase substrate, such inhibitory compounds should bear both a double bond between carbon 2 and 3 and a hydroxy group on carbon 3 like quercetinase substrates.

Finally various compounds acting as more or less specific inhibitors of metalloenzymes such as sodium azide, potassium cyanide, EDTA, hydrogen peroxide, *p*-chloromercuribenzoate, *N*-ethyl maleimide, ascorbate and iodoacetamide were not inhibitors of *A. flavus* quercetinase (Oka et al. 1972).

## The dioxygenase nature of quercetinase

The necessary presence of dioxygen for the degradation of rutin or quercetin in culture filtrates (containing rutinase, quercetinase and esterase activities) of *A. flavus* grown on rutin was recognised very early (Westlake et al. 1959). Nearly 10 years later (Krishnamurthy and Simpson 1970), the incorporation of dioxygen into the products of the reaction (CO and the depside) was investigated with labelled dioxygen ( $^{18}\text{O}_2$ ). A purified quercetinase from *A. flavus* was used and quercetin was placed in either  $\text{H}_2^{18}\text{O}/^{16}\text{O}_2$  or  $\text{H}_2^{16}\text{O}/^{18}\text{O}_2$  incubation mixtures. In the two cases, the evolved CO was not marked nor was the depside in the former case, indicating that no water was involved in the transformation of quercetin. In the case of labelled dioxygen, when the depside (as its tetramethyl ether) was analysed by mass spectrometry, the incorporation of two  $^{18}\text{O}$  atoms was evidenced. Furthermore the two O atoms were found to be located in the two carbonyl functions of the depside (by mass spectroscopy, infrared spectroscopy and  $\text{CO}_2$  analysis after decarboxylation of the depside). These results suggested that quercetinase is a dioxygenase and that carbons no. 2 and 4 were the sites of dioxygen incorporation in the flavonol substrate, leading to the conclusion that cyclic peroxide was necessarily formed during quercetinase catalysis. This was confirmed later on (Brown et al. 1982) by using the same enzyme, quercetin and various  $^{16}\text{O}_2/^{18}\text{O}_2$  proportions and analysing the incorporation of labelled oxygen atoms into the depside. By comparing the experimental heavy oxygen atom incorporations to the theoretical ones, the authors came to the conclusion that both O atoms arose from a single dioxygen molecule.

## EPR characterisation of quercetinase

It has already been mentioned that *A. niger* quercetinase was characterised by EPR, leading to the conclusion that the enzyme possessed a non-blue type II  $\text{Cu}^{2+}$  atom in a distorted square planar geometry. Anaerobic quercetin addition in threefold excess yielded no spectral changes (Hund et al. 1999).

A detailed EPR study of the heterologously expressed and purified quercetinase of *A. japonicus* was published soon after (Kooter et al. 2002). By atomic absorption, the copper content of the

preparation was shown to be 0.8 eq. The EPR spectrum of native quercetinase at pH 6 was clearly indicative of the presence of two species. The major form possessed a spectrum close to those of type 2 copper sites with a  $g$  tensor of nearly axial symmetry, the minor form showing a more distorted geometry. When the pH was shifted from 6 to 10, single specie was characterised closely related to the pH 6 minor form. This change was reversible and by simulation, trigonal bipyramidal geometry was assigned. Anaerobic incubation of quercetin with native quercetinase resulted in a totally new EPR spectrum corresponding to single species. By spin quantification, the addition of quercetin and its binding to copper was shown to occur without effect on copper oxidation state ( $\text{Cu}^{2+}$ ). Exposure to air of the previous sample lead to a new specie still different from the native enzyme by EPR, unless extensive dialysis was realised, indicating a bound compound. This compound was found to be the product of the reaction (i.e. the depside), as it gave the same EPR spectrum when incubated with quercetinase. It should be noted that CO has no influence in this context. Eight other flavonols (galangin, kaempferol, myricetin, morin, datisatin, fisetin, 7-hydroxy flavonol and flavonol) were also anaerobically incubated with quercetinase producing well defined EPR spectra. The similarity of these spectra with the one obtained for quercetin lead to the conclusion that the arrangement of the copper ligands was similar in all cases. To the contrary, the addition of a flavone (apigenin), a flavonol (taxifolin) and a flavan-3-ol (epicatechin) did not change the native enzyme EPR spectrum, something indicative of the absence of bonding. This result is in accordance with the strict substrate specificity of quercetinases for flavonols, highlighting the necessity of a 3-OH group, a 4-keto group and a C2–C3 double bond allowing thus a planar geometry of the substrate.

In another EPR study (Fittipaldi et al. 2003) done on a single crystal of *A. japonicus* quercetinase binding diethyldithiocarbamate, the complete  $g$ -tensor of the copper centre has been determined.

#### Structural characterisation of quercetinase

Highly valuable structural informations on quercetinase came from X-ray spectroscopic experiments on native or recombinant *A. japonicus* quercetinase

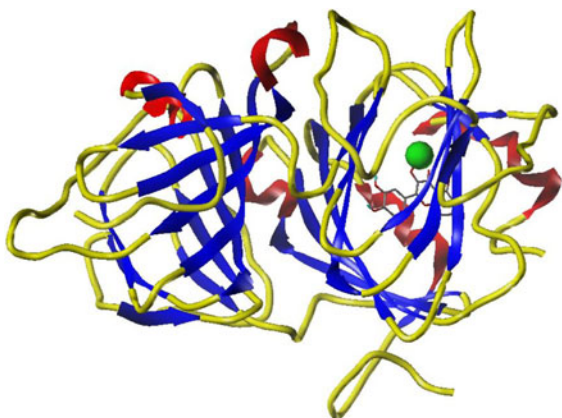
(Fusetti et al. 2002; Steiner et al. 2002a, b, c) either free or complexed with substrates or inhibitors.

#### *X-ray diffraction study of the free enzyme*

The first precise structural information on quercetinase came from the X-ray diffraction study of partially deglycosylated native *A. japonicus* quercetinase (Fusetti et al. 2002). The enzyme crystallised in space group P2 with four molecules forming two homodimers in the asymmetric unit. Five potential Asn-X-Thr glycosylation sites were found, all of them bearing at least one N-acetyl glucosamine (Asn 90, 109, 142, 191, 248). Furthermore well defined branched heptasaccharide was visible at Asn 191 which could be involved in oligomerisation.

Each monomer is formed by a N-terminal domain (residues 1–145), a C-terminal domain (residues 206–350) and a linker (residues 146–205). The two domains are similar, sharing about 20% sequence identity, and positioned face-to-face around a pseudo 2 fold symmetry axis (Fig. 9).

Each domain is formed by two short  $\alpha$ -helices and two antiparallel  $\beta$ -sheets composed of eight strands forming a  $\beta$ -sandwich. In the catalytic site, located in the N-terminal domain, a supplementary  $\beta$ -sheet is present. The copper atom is found only in the N-terminal domain since the three histidine copper ligands were replaced by other aromatics in the C-terminal domain. In the two domains a hydrophobic pocket was found. From amino acid sequence and three dimensional structure comparisons, quercetinase could be classified in the recently recognised cupin superfamily (Dunwell 1998; Dunwell and Gane 1998; Dunwell et al. 2000, 2001, 2004; Clissold and Ponting 2001; Mills et al. 2002; Agarwall et al. 2009) and more precisely in the bicupin sub-group due to the presence of two cupin domains (N- and C-terminal domains). The two consensus sequences  $\text{PG}-(\text{X})_5-\underline{\text{HXH}}-(\text{X})_4-\underline{\text{E}}-(\text{X})_6-\text{G}$  and  $\text{G}-(\text{X})_5-\text{PXG}-(\text{X})_2-\underline{\text{H}}-(\text{X})_3-\text{N}$  separated by 15–50 amino acids, which are the cupin signature, were found more or less altered in the two domains. The cupin family is composed of a lot of metalloproteins whose metal are complexed by the three histidines and the glutamate of the two consensus sequences (underlined in the consensus sequences, see above). By structural comparison, *A. japonicus* quercetinase was related most closely to germin (a monocupin Mn containing oxalate oxidase)



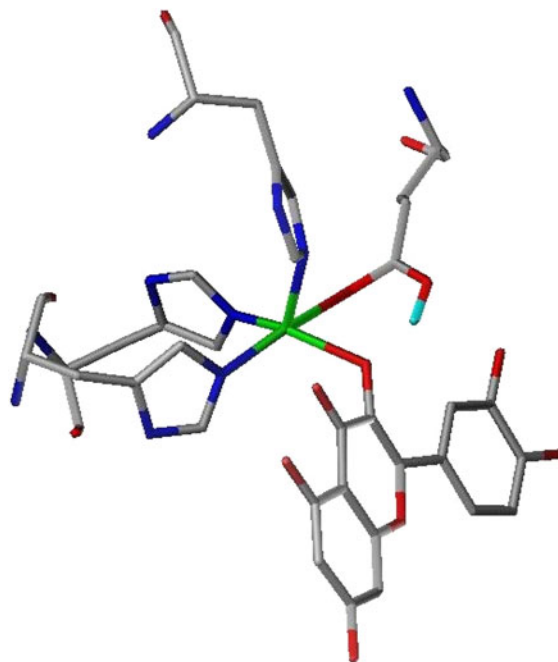
**Fig. 9** The mono metallated bicupin nature of *A. japonicus* quercetinase (PDB code 1h1i)

and homogentisate dioxygenase (a bicupin Fe containing dioxygenase). Interestingly two modes of copper coordination were found in *A. japonicus* quercetinase in close agreement with EPR measurements (see above, Kooter et al. 2002). The major one (70%) consisted of a distorted tetrahedral geometry around copper with His66, 68 and 112 and a water molecule acting as ligands. In the minor form Glu73 acted as a supplementary ligand leading to trigonal bipyramidal geometry His66 and Glu73 being the axial ligands (Fig. 10).

#### *On the bicupin nature of mould quercetinases*

The recently published *P. olsonii* quercetinase sequence (EU126643) prompted the authors (Tranchimand et al. 2008) to compare it to an other known quercetinase sequence (*A. japonicus*) and to potential quercetinase sequences (*A. oryzae*, *A. niger* and *A. nidulans*) retrieved from the database after a BLAST search (Fig. 11).

Using *P. olsonii* quercetinase sequence as reference, 68, 63, 61 and 58% sequence identity were found for *A. oryzae*, *A. nidulans*, *A. niger* and *A. japonicus* quercetinases respectively. From the sequence alignment it could be concluded that all these true and putative quercetinases belong to the cupin superfamily and to the bi-cupin subgroup. The canonical metal complexing tetrad (3 His, 1 Glu) is only found in their N-terminal domain, perfectly conserved, establishing these proteins as mono-metallated bi-cupins. For all these proteins, the loop linking the two cupin domains is approximately of the same length and remarkably



**Fig. 10** Copper coordination in *A. japonicus* quercetinase site (after Steiner et al. 2002a)

the Pro164 (*A. japonicus* numbering) supposed to play a great role in the locking of the substrate in the active site (Steiner et al. 2002a) is perfectly conserved. Moreover amino acids involved in the substrate binding pocket of *A. japonicus* quercetinase (Steiner et al. 2002a) are all perfectly conserved (Tyr35, Met51, Thr53, Phe75, Phe114, Met123, Ile127, *A. japonicus* quercetinase numbering) as are three (Phe132, Phe135, Phe136, *A. japonicus* numbering) of the four amino acids predicted to form a dioxygen channel in *A. japonicus* quercetinase (Fiorucci et al. 2006). It was therefore concluded that all these quercetinases belong to a class of enzyme highly conserved in Ascomycetes.

#### *X-ray absorption studies*

XANES analysis of the enzyme, free or anaerobically complexed with its substrates quercetin or myricetin, reveals interesting features (Steiner et al. 2002b). First of all the complexed enzyme retains the formal initial  $\text{Cu}^{2+}$  oxidation state of the native enzyme, an important point for mechanistic considerations. Then the anaerobic addition of either of the two substrates

<i>P. olsonii</i>	1	MLTSALFLLASLGLAQSATLEDLYLENAPSAPRPVVI	PHYANSHAVAVGDQVYRFTVTGPSSDNAFTLM	STNAPSSSSLG	80
<i>A. oryzae</i>	1	M-NLLLLAGLLPLAASQSVSDLYLENAPSSRPYII	PHYANSHAVTIGSQLYRFPVTVGPSSDYAFTLM	STNAPYSGSLG	78
<i>A. nidulans</i>	1	M-VALNLLLAATPLAAAQSLSDLYLDSAPSSRPYI	IIPHYANSHAVTIGAQLYRFPVTVGPASDYAFTLM	STNAPASGSLG	79
<i>A. niger</i>	1	M--RSFVFSLLALTSALSVEDLYVSEAPSSRPYI	ILPHYENSHAVTIDSQLYRFTVTGPSSDYAFTLM	STNAPSSSSLG	78
<i>A. japonicus</i>	1	DTSSLIVEDAPDHVRPVVIRHYSHARAVTVDTQL	YRFYVTVGPSSGYAFTLMCTNAPHSDALG		62
		* * * * *	* * * * *	* * * * *	
<i>P. olsonii</i>	81	VLPHVHQKHYLENFFSFKGRFQLWAQKDAEQARLLT	AGDYGSVPRNTTHTEQILDPDTEMVGVITVPGGFEDL	FYALGTN	160
<i>A. oryzae</i>	79	VLPHTHQKHYLENFFNFKGRFQLWAQYQGEEOQGR	LLTQGDYGSVPRNTTHTEQILDPDTEMVGVITV	SPGGFEDLFYTLGTN	158
<i>A. nidulans</i>	80	VLPHTHQKHYLENFFNFKGRFQLWAQKGDGEQEAR	LLTQGDYGSVPRNTTHTEQILDPDTEMVGVITV	SPGGFEDLFYALGTN	159
<i>A. niger</i>	79	VLPHTHQKHYLENFFSFKGRFQLWAQKGDGEQEAR	LLTQGDYGSVPRNTTHTEQILDPDTEMVGVITV	PGGFEDLFYALGTN	158
<i>A. japonicus</i>	63	VLPHTHQKHYLENFCYCKGSPQLWAQSGNETQQTR	VLSSGDYGSVPRNVTHTEQIQDPDTEMVGVITV	PGGFEDLFYALGTN	142
		*** * * * * * * * * * * * * * * * * * * *	* *		
<i>P. olsonii</i>	161	YTSRDTGTPYVPAHNS--SSSS- <b>PDSSV</b> ISSLQK	FVDVYAKLGFEPDRDLVNGTAPVDNSEWHTGDNA	---LGT	233
<i>A. oryzae</i>	159	YTSATNTPYVPQASN--DSSAT <b>GP</b> DASGISGLE	SFVDVYAQLSFEPDRDFVNGSAPSDEG-WHTEDAS	---LGE	232
<i>A. nidulans</i>	160	YSDATNTPYVPAASN--GTSS-- <b>PGADV</b> ISSLQK	YDVYAQLDFEPTDRDLVNGSSSTSS-WHAIDSSA	TPLEG	234
<i>A. niger</i>	159	YTSGTETPYVPGSGNSSSSSAT <b>GP</b> DSSITISGL	QQYDVYAQLDFTPRRDIVNGSAPSDSG-WHTESD	---LGA	234
<i>A. japonicus</i>	143	ATD <sup>o</sup> THTPI <sup>o</sup> IPSSD--SSST <b>TG</b> DSSTISTLQSPD	VYAELSFPTPRDTVNGTAPANTV-WHTGANA--	LASTAG	217
		* * * * *	* * * * *	* * * * *	
<i>P. olsonii</i>	234	ANGYGPKYLNSKFG-YQVVQPFVTAKQAQDTN	TYLTLSTISLNLH-QTKNNAPSYTLKGATAFEVIE	GLVKI	311
<i>A. oryzae</i>	233	ANNYGPKYLNSKHGAYQIIQPLVNTNSQAQDTN	FLLSTIILSRQRTSSSAPTWSVTGATAFEVIEGS	VQI	312
<i>A. nidulans</i>	235	ANNYGPKYLNSRHGNYQIVQPLVTNAQSDTN	FLLSTIVMSR-QHSEMVDTWKANGACAFEVIEGS	VLIKIGAY	313
<i>A. niger</i>	235	ANNYGPKYLNSQYGAQYQIVQPLVTATQAQDTN	TYLTLSTIIMSRQPSNATAPWTWAG-PAAFEVIE	GSVQVQIG	313
<i>A. japonicus</i>	218	ANGWPKYLNSQYG-YQIVAPFVVTATQAQDTN	TYLTLSTISMSTTPTSTVTPWTSFPGACAFQVQ	EGRVVVQIGD	313
		** * * * * * * * * * * * * * * * * * * *	* * * * * * * * * * * * * * * * * * * *		
<i>P. olsonii</i>	312	TGDVAFVPAGVSFTYYTEVAFTKALYIASGNSG	VDSQLIQGGKTNWNVTFPTQ		364
<i>A. oryzae</i>	313	MGDVAFVPAVSYSYWTEGSYAKVLSVNAQDGLN	QQLIAEGKDWGYPTFPRY		365
<i>A. nidulans</i>	314	TGDVAFVPKNTAFRYWTTGSYAKVLYVSAGQE	GLDKRLIERGENWSYPTFPKSWKKTTEL		372
<i>A. niger</i>	314	MGDVAFVPKGVTYRYWNEGAQAKVLYWRRVG	-----VMEIQHVAGPRI	PGCGGGLG	364
<i>A. japonicus</i>	297	SGDVAFIPGGVEFKYYSEAYFSKLVLFVSSSGD	LDLQNLVNGGEEWSSVSFPADW		350
		*** * * *	*		

**Fig. 11** Alignment of characterized and putative quercetinases from the ascomycetes *P. olsonii* (EU126643), *A. oryzae* (BAE-62701), *A. nidulans* (XP664376), *A. niger* (XP001388596) and *A. japonicus* (Q7SIC2). Domain 1 (aa 1–145) and 2 (aa 206–350) (Fusetti et al. 2002) are boxed in gray; copper coordinating aa H66, H68, E73, H112 (Fusetti et al. 2002) are boxed in white; aa involved in the substrate binding pocket Y35, M51, T53, F75,

F114, M123, I127 (Steiner et al. 2002a) are boxed in black; peptide 154–169 in the *A. japonicus* sequence is shadowed; P164 that close the active site upon substrate binding (Steiner et al. 2002a), is in bold; aa predicted to form a dioxygen channel (Fiorucci et al. 2006) are marked with a filled circle (after Tranchimand et al. 2008)

was shown to induce a similar change in the copper environment when compared to the native enzyme. This point was confirmed upon EXAFS studies. By simulation of the obtained EXAFS spectra, the coordination of the copper atom could be equally well described as tetracoordinate (3 N and 1 O) or penta-coordinate (3 N, 2 O) in the native enzyme. Upon anaerobic addition of either quercetin or myricetin, copper coordination is best described as pentacoordinate (3 N, 2 O). In that case the two oxygen ligands are Glu73 and the substrate flavonol, both linked in a monodentate mode. In all these studies the coordination mode of the three histidines is invariant whatever the status of the enzyme (free or complexed).

*X-ray diffraction studies of complexed quercetinase*

The fate of coordination change upon binding was confirmed when the structures of *A. japonicus* quercetinase complexed with either its inhibitor DDC

(diethyldithiocarbamate) or kojic acid (a structural analogue of flavonol ring C lacking both A and B rings) were solved. With DCC the two sulfur atoms are bound asymmetrically to the copper atom in a distorted square pyramid geometry, Glu73 not acting as a ligand. Upon kojic acid ligation the copper coordination became pseudooctahedral with Glu73 now acting as a ligand, as do kojic acid in a bidentate fashion, through both O3 (C3–OH) and O4 (C4=O) oxygen atoms (Steiner et al. 2002b).

The most biologically relevant structural information came from the solved structure of *A. japonicus* quercetinase complexed anaerobically with its substrates quercetin and kaempferol (Steiner et al. 2002a). The two substrate-complexed structures were found similar but differed from the one of native enzyme by the fact that the linker region was now correctly solved, drawing a lid closing the active site. The linker is constituted of two helices and proline 164 is supposed to established van der Walls interactions



with the A ring of the flavonols. Of great importance was the fact that the substrates were ligated to copper in a monodentate fashion through their O3 (C3–OH) oxygen atoms. Copper was therefore pentacoordinated, His66, His112, Glu73 and O-3(fla) forming the base of the square pyramide and His68 being the apical ligand. Some distortion toward trigonal bipyramide was also observed. The substrate was also stabilised as a result of the situation of its B ring in a hydrophobic pocket establishing van der Waals interaction with various residues side chains (Tyr35, Met51, Thr53, Glu73, Phe75, Phe114, Met123, Ile127) and backbone atoms of Gly125. The A and C rings were also involved in van der Waals interactions. Only two hydrogen bonds were involved between water molecules and O7 and O4' oxygen atoms of the flavonols. All these structural characteristics are summarised in Fig. 12.

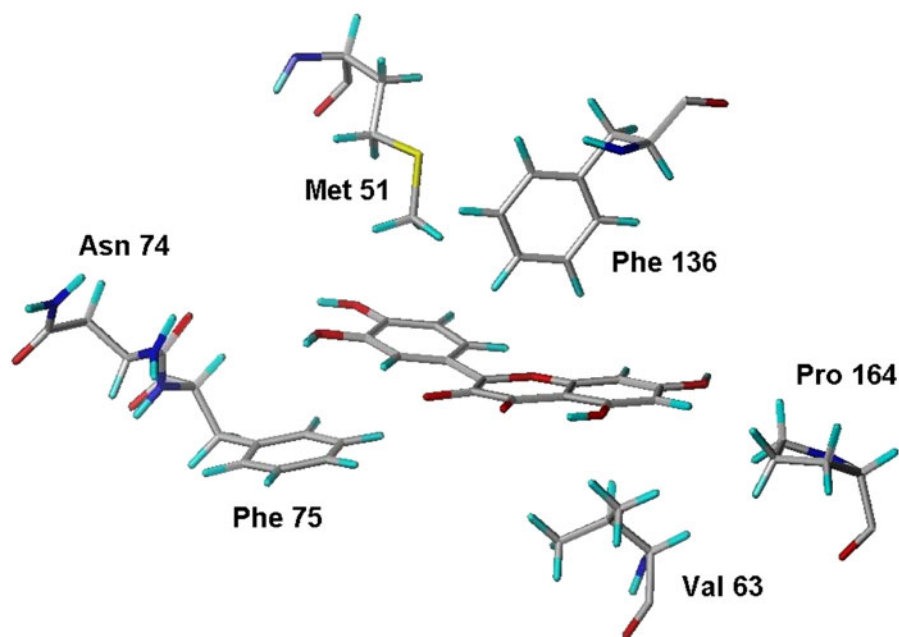
Another important point was revealed by this structural study. Indeed when packed in the active site, quercetin and kaempferol which are planar molecules, had their B-ring bent out of the plane of the molecule by 10–12°. This corresponded to a pyramidalisation of the C2 atom enhancing its sp<sup>3</sup> character. This particular flavonol conformation was shown to cost only 1 kcal/mol by DFT calculations and looked to be the result of steric repulsion of the B ring due to the side chains of Phe75 and Phe114 and possibly to CH– $\pi$  interaction between the methyl group of Met51 and the B ring of the substrate.

#### *Insights from molecular dynamics simulations and quantum calculations*

A specific study on the dynamic of the linker region and of the substrate cavity of the quercetinase from *A. japonicus* has been conducted through molecular dynamics simulations (van den Bosch et al. 2004). The obtained results were in very good accordance with X-ray diffraction studies (Fusetti et al. 2002; Steiner et al. 2002a). Indeed, without substrate in the active site the loop, being part of the linker region between N-terminal and C-terminal domains and which is close to the substrate cavity, possessed a large mobility. This mobility is strongly reduced upon substrate binding, reinforcing the idea that the loop acted to lock the substrate in the active site. Following loop motion, interaction between Pro164 and the substrate and supplementary hydrogen bonds

between the loop and the rest of the protein are formed at expense of hydrogen bonds between the loop and the substrate. Similar results were reported later (Fiorucci et al. 2007) showing that the linker region could be divided in sub-domains showing either high flexibility whether or not the substrate is present in the cavity (residue 146–153 and 170–199) or showing flexibility only in the absence of substrate (residue 154–169 and 200–204). Another molecular dynamics study focused on the substrate cavity (Fiorucci et al. 2006). Starting from the free enzyme crystalline structure, water entrance was shown to occur (from eight in the solved structure (Fusetti et al. 2002) to 16–18 at the end of the simulation) leading to an increased volume of the cavity of more than 100% (from 240 to 520 Å<sup>3</sup>). The same phenomenon was already observed in the previous simulation (van den Bosch et al. 2004) with a final 13 water molecules filling the substrate cavity. But in the latter, the starting structure was the one crystallised with the substrate and the simulation was started after removal of the substrate. Another important feature was established by molecular dynamics (Fiorucci et al. 2006). The dynamic of water entry revealed the presence of a water canal linking the substrate cavity and the outside of the protein. Indeed during water entrance, Leu135 side chain rotated opening the middle of the canal and Phe175 side chain flipped from the surface of the protein to the bulk solvent, opening a large gate to the substrate cavity. The side chain of Phe132 is also involved in the canal formation. Also filled by water during the simulation, its hydrophobic character raised the question whether this canal could be dedicated to dioxygen travelling. Another simulation came to the conclusion that the canal is perfectly suited for small molecule diffusion, especially dioxygen, without important energetic constrain (less than 2.5 kcal/mol). At the end of the canal, it was found that dioxygen could interact with either copper (10%) or quercetin on the C2 carbon atom (90%), a key point from a mechanistic point of view. The opening of the channel was also shown to be dependant of an increase of the substrate cavity of the *A. japonicus* quercetinase (initially crystallised with or without substrate) following either water or substrate entrance respectively during the simulation (Fiorucci et al. 2007). Other simulations have led to the conclusion that the main points concerning enzyme–substrate interactions already observed in

**Fig. 12** Substrate interactions in *A. japonicus* quercetinase active site (after Steiner et al. 2002a)



substrate-complexed crystallised *A. japonicus* quercetinase (Steiner et al. 2002a) could also be valid in solution: (i) monodentate copper chelation of the substrate, (ii) hydrophobic interaction between substrate and side chains of Met51, Val63, Phe75 and Phe136, (iii) hydrogen bonds between two conserved water molecules and C7–OH and C3'– and C4'–OH from the substrate quercetin (Fiorucci et al. 2007). Estimated free energy binding of quercetin within quercetinase were also computed leading to quite high values ranging from  $-35.4$  to  $-39.4$  kcal/mol showing thus that quercetin is strongly bound to quercetinase before catalysis (Fiorucci et al. 2007).

In an effort to explain experimentally determined rate of degradation of various flavonol during *A. flavus* and *P. olsonii* quercetinase catalysis (Oka et al. 1972; Tranchimand et al. 2008), in relation with their B ring substitution patterns, quantum calculations were very recently conducted (Antonczak et al. 2009). Although this study does not give a definitive answer to explain the observed relative degradation rate ranking (kaempferol > quercetin > myricetin > galangin), some key points relevant to quercetinase catalysis were put forward or reassessed. No special polarisation of the flavonol when present in the substrate binding pocket was noticeable except for atoms present in the first coordination sphere of the copper center. The rate-limiting step of quercetinase was found to be the formation of the cyclic

endoperoxide, bridging carbon 2 and 4. The substrate is tightly anchored in the catalytic pocket through three points attachment (hydrogen bonds with two water molecules present in the catalytic cavity and copper chelation through the deprotonated flavonol O3 atom). The role of protonated glutamate copper ligand playing a role for substrate activation (deprotonation) but also during catalysis by establishing hydrogen bond with one of the atom of the dioxygen molecule either free or already ligated to the substrate. The importance in substrate activation of the rotation of the B ring of the substrate out of the plan of the fused A–C ring system and the related pyramidalisation of the C2 carbon atom of the substrate. The former phenomenon is used to explain the relative ranking of degradation rate kaempferol > quercetin > myricetin. Indeed the rotation of B ring is favoured in the case of kaempferol since this flavonol bears a unique hydroxy substitution on B ring that is placed in the axis of the rotation while quercetin and myricetin have respectively one and two supplementary hydroxy substitution that energetically impaired the rotation of their B ring.

#### Mechanistic aspects of quercetinase catalysis

In the precedent chapters various mould quercetinase characteristics have been reviewed. Numerous of these findings have been used by various authors to

propose a mechanism for quercetinase catalysis. These proposals have also been evaluated by theoretical studies trying to explain some of the experimental results described to date for quercetinase catalysis. A remainder of the most important features in relation with mechanistic considerations will be discussed in the following and most plausible mechanisms for quercetinase catalysis will be presented.

#### *DFT studies*

Recently two DFT studies were dedicated to mechanistic aspects of quercetinase catalysis (Siegbahn 2004; Fiorucci et al. 2004). In the two cases the role of copper was found to activate the substrate after its deprotonation [arising from hydrogen abstraction by Glu73 (*A. japonicus* numbering)] through oxidation of the formed flavonolate ion leading to a formal Cu<sup>I</sup>-flavonoxy radical complex. This activation is necessary to explain the spin forbidden reaction that is catalysed by quercetinase, a single state molecule of substrate reacting with a triplet state dioxygen molecule leading to triplet state products. At this point the dioxygen could add on either the copper ion or on the C2 carbon atom of the substrate. It was shown (Siegbahn 2004) that dioxygen addition on copper is favoured over addition on substrate, leading thus to a copper-hydroperoxy radical. The latter then evolved to a bridged peroxide by addition on the C2 carbon atom of the substrate. The Cu–O bond was then lost and the formed peroxy anion attacked the C4 carbonyl atom, allowing the bonding between the now Cu<sup>II</sup> ion and the alcoholate ion located on C4. Then the cleavage of one O–O and two C–C bonds lead to the formation of CO and deprotonated depside product which reprotonated from Glu-OH73. In the other study (Fiorucci et al. 2004) the dioxygenolysis of quercetin was considered in a larger sense, not restricted to quercetinase catalysis. Addition of dioxygen was envisioned on either activated or non-activated quercetin. The addition of dioxygen on activated quercetin (deprotonated and oxidised) was shown to be favoured versus addition on only deprotonated quercetin. The C2-hydroperoxy thus formed was then considered to evolve either by a 1,2-cycloaddition leading to a dioxetan or by a 1,3-cycloaddition leading to an endoperoxide. The dioxetan formation was shown to be energetically favoured but it should be noted that the further evolution of the dioxetan could not lead to

the expected products of the quercetinase catalysed reaction. In conclusion the two studies highlighted the role of deprotonation of the substrate and of copper mediated oxidation of the substrate leading to a Cu<sup>I</sup>-flavonoxy radical complex which could then add dioxygen either on the metal or on the substrate.

#### *Experimental points relevant to quercetinase catalysis*

First of all the two products of the reaction catalysed by quercetinases are carbon monoxide and a depside arising only from flavonols. Various mould quercetinases were found to contain at least one copper atom in the cuprous resting state. The use of copper chelating quercetinase inhibitors has proved that copper is catalytically active. This copper atom is believed to allow the activation of the substrate, but upon anaerobic binding of the substrate the copper oxidation state is mainly unchanged. Numerous biomimetic studies have highlighted that the initial step of the catalysis is the probable coordination of the substrate to the copper ion [for a general account on biomimetic studies of quercetinase action see (Kaizer et al. 2006)]. Furthermore using reductants, only the cuprous state was shown to be catalytically active. By using labelled dioxygen, the two oxygen atoms were found in the depside product both arising from the same dioxygen molecule establishing thus the dioxygenase nature of quercetinase. It was also shown that carbon atoms C2 and C4 were the sites of dioxygen incorporation in the flavonol substrate leading to the conclusion that a cyclic peroxide is necessary formed during quercetinase catalysis. On the other hand the carbon atom of carbon monoxide was shown to arise from the C3 carbon of the substrate (Krishnamurty and Simpson 1970). X-ray diffraction studies have shown that the substrate binds the copper atom in a monodentate fashion through its O3 atom. This binding was thought to occur after deprotonation of the C3–OH group. The only residue in the active site able to act as a base is Glu73, a residue also implicated in copper chelation. Substrate chelation has also been proved to modify the copper coordination from a 3 N, 1 O tetrahedral or bipyramidal trigonal geometry to a 3 N, 2 O square pyramidal geometry. As a result of substrate binding, a flavonolate–Cu<sup>II</sup> complex is formed which could

evolve to a flavonoxyl–Cu<sup>I</sup> radical. Indeed upon substrate binding in the active site of *A. japonicus* quercetinase, the pyramidalisation of the C2 carbon atom of the substrate was revealed by X-ray diffraction study, enhancing thus its sp<sup>3</sup> character as well as its spin density. Nevertheless the enzyme–substrate complex was found to be mainly in the flavonolato–Cu<sup>II</sup> form. In order to overcome the spin barrier of the reaction catalysed by quercetinase, the tautomeric form of the latter should be formed (i.e. the flavonoxyl–Cu<sup>I</sup> radical), although the thermodynamic equilibrium is not in favor of this form. The flavonoxyl–Cu<sup>I</sup> radical is supposed to be the activated enzyme–substrate complex that could react with dioxygen and is also believed to be formed in quite low quantities. But in presence of dioxygen this enzyme–substrate complex is probably quickly quenched by dioxygen. At this point two sites for dioxygen attack are available either on the Cu<sup>I</sup> atom or on the C2 substrate carbon atom acting as a radical. DFT calculation has proved that dioxygen addition on Cu<sup>I</sup> is favoured. The presence of a dioxygen channel, revealed by molecular dynamics simulation, could deliver dioxygen on either Cu (10%) or C2 substrate carbon atom (90%) but did not give a clear cut answer, although it favours the initial formation of a flavonolato hydroperoxide radical. But whatever the initial site of dioxygen attack the formation of an endoperoxide bridging copper and the C2 carbon atom of the flavonol could be proposed. This endoperoxide could then rearrange leading to a pure organic endoperoxide bridging that time the C2 and C4 carbon atoms of the substrate. This rearrangement could occur through the transient formation of a C2 hydroperoxide anion following copper oxidation (Cu<sup>I</sup> → Cu<sup>II</sup>). It should be noted that various authors, when considering the initial attack of dioxygen on the C2 carbon atom of the substrate do not propose the next formation of an endoperoxide bridging copper and the flavonol but favours the direct attack of the peroxide radical on the C4 carbon atom of the substrate. The collapse of the formed C2–C4 endoperoxide intermediate generated then both CO and a deprotonated depside bound to copper. The depside is finally reprotonated from Glu73-OH, leaving then the active site. A summary of two of the plausible mechanisms of quercetinase action described thereof is presented in Fig. 13.

## Other quercetinases

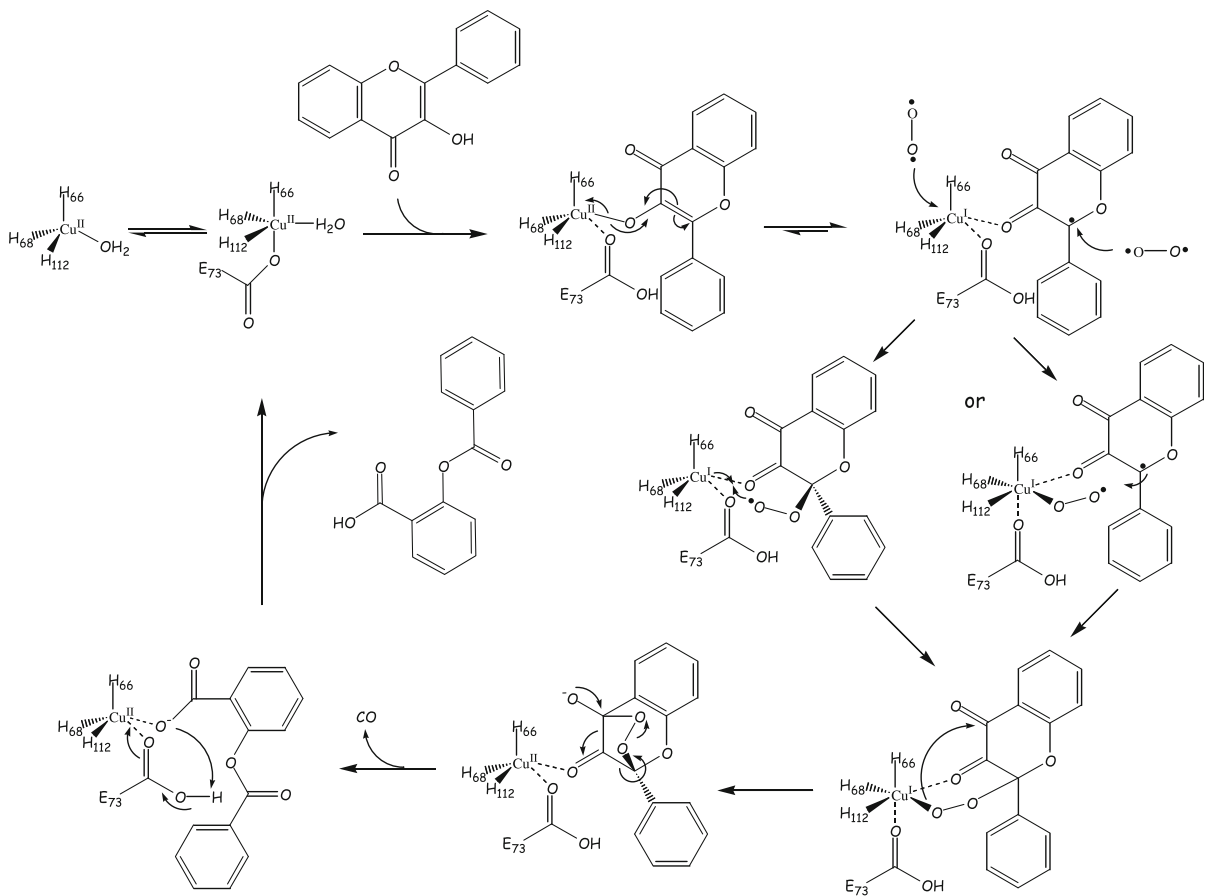
The archetypal quercetinases are actually considered to be those produced by moulds and involved in flavonol catabolism. Recent studies have identified quercetinase in two bacterial species [*Bacillus subtilis* (Bowater et al. 2004; Barney et al. 2004) and *Streptomyces* sp. (Merkens et al. 2007)], but no clear physiological role was assigned to these two enzymes. Beside a catabolic role similar to the one ascribed to eukaryotic quercetinase, a detoxification role towards potentially deleterious effects caused by flavonols when present inside the bacteria could be envisioned. Another protein, pirin, found in numerous living species (ranging from *E. coli* to *Homo sapiens*) has been shown to catalyse the same reaction as mould quercetinases, although its main function is thought to be as a co-transcriptional factor. As for the two bacterial enzymes, a role in flavonol detoxification process has also been postulated for the quercetinase activity of pirins (Adams and Jia 2005). Some characteristics of these three enzymes are discussed in the following.

### *Bacillus subtilis* quercetinase

#### *Biochemical characterisation of Bacillus subtilis quercetinase*

In 2004, two articles (Bowater et al. 2004; Barney et al. 2004) reported the heterologous expression in *E. coli*, of the YxaG protein from *Bacillus subtilis*, in a functional genomic optic. The function of the YxaG protein was first postulated after BLAST search as being quercetinase. Indeed the second most related protein sequence was the one of *A. japonicus* quercetinase. The BLAST search was also indicative of the probable bimetalated bicupin nature of YxaG since the two cupin domains were shown to possess the four canonical metal coordinating ligands (3 His, 1 Glu) unlike eukaryotic quercetinases. The two groups overexpressed YxaG in *E. coli* with (Bowater et al. 2004) or without (Barney et al. 2004) a His-tag. In the former case the purification was done by a single metal affinity chromatography step.

The reaction catalysed by YxaG was shown to be the same as for eukaryotic quercetinase using quercetin as substrate (Bowater et al. 2004; Barney et al. 2004). Indeed both CO and the depside were identified



**Fig. 13** Summary of two of quercetinase plausible mechanisms of action. Phenolic substitutions have been omitted for clarity

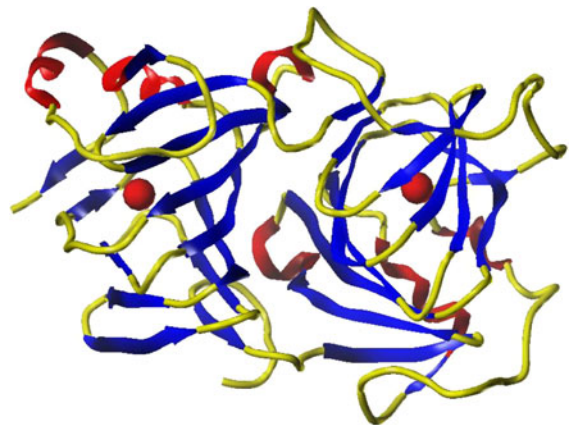
with a substrate/CO ratio of 1 (Bowater et al. 2004). The optimum pH of the enzyme was found to be higher than for eukaryotic quercetinases (7.5 vs. 6, Barney et al. 2004). The two studies delivered similar kinetic parameters, using quercetin as substrate [ $K_m = 3.8 \mu\text{M}$ ,  $V_{\text{max}} = 1.2\text{--}2 \text{ U/mg}$  (Bowater et al. 2004) and  $K_m = 0.8 \mu\text{M}$ ,  $V_{\text{max}} = 1.5 \text{ U/mg}$  (Barney et al. 2004)]. It should be noted that if the  $K_m$  values were comparable to those found for eukaryotic quercetinases, the  $V_{\text{max}}$  values were found to be two order of magnitude lower ( $V_{\text{max}} = 180 \text{ U/mg}$  for *A. flavus* quercetinase), raising doubt about the true substrate of YxaG protein (Barney et al. 2004). The molecular mass of the overexpressed prokaryotic quercetinase was determined by MALDI-TOF mass spectrometry and prove to be reasonably close to the theoretical expected values of 37,600 and 38,660 kDa (with or without tag respectively). The enzyme was found to be dimeric in solution (Bowater et al. 2004) and totally insoluble at pHs lower than 5 (Barney et al. 2004). The

metal content was analysed by the two groups and if iron was found in highest amounts other metals such as Zn, Cu, Mn and Ni were also detected. The iron equivalent per enzyme monomer (two cupin sites per monomer) were different in the two studies [1.7–2.2 eq. (Bowater et al. 2004) and 0.5–0.8 eq. (Barney et al. 2004)]. X-band EPR spectroscopy was found to be consistent with the presence of a high spin  $\text{Fe}^{\text{III}}$  specie with a resonance at  $g = 4.31$  (Bowater et al. 2004; Barney et al. 2004). But iron bound to the protein was shown to be mainly in the  $\text{Fe}^{2+}$  EPR silent oxidation state. Indeed with NO as  $\text{Fe}^{\text{II}}$  probe, ferrous ion was detected (Barney et al. 2004). The role of iron was also highlighted by the use of inhibitors possessing metal chelating properties. Iron chelators such as 1,10-phenantroline and 2,2'-bipyridyl acted in the micromolar range while copper chelates such as ethyl xanthanate and diethyldithiocarbamate acted only in the millimolar range (Barney et al. 2004).

### Structure of *Bacillus subtilis* quercetinase

The structure of YxaG protein heterologously expressed in *E. coli* was solved at 3.5 Å resolution by an X-ray diffraction study (Gopal et al. 2005). The crystal structure revealed a compact arrangement of the two cupin domains as well as the formation of homodimers in the crystal structure (Fig. 14). It should be noted that the bicupin fold of *Bacillus subtilis* quercetinase is quite robust since after ca. 33% of proteolytic cleavage (trypsin), it maintains high levels of activity, thermal stability and overall structure organisation (Rajavel et al. 2008).

Each coordination site of the cupin domains was filled with an Fe<sup>II</sup> atom. The N- and C-terminal cupin domains were joined by a flexible linker of 18 residues, were related by a two-fold axis of symmetry and could be superimposed with a rms deviation of 1.11 Å. The interface between the two cupin domains was important (3,100 Å<sup>2</sup> surface), 72% of which being constituted of non-polar residues. All these structural features were similar to those described for *A. japonicus* quercetinase (Fusetti et al. 2002; Steiner et al. 2002a, b, c). Modelling of quercetin in the N-terminal active site occurred without major steric hindrance. When compared to *A. japonicus* quercetinase, the active site was shown to be larger, due to the replacement of bulky substituents in the eukaryotic enzyme by smaller ones in its prokaryotic counterpart. Some differences were also noticed when the iron geometry was compared in the two cupin domains of YxaG. This was mainly due to the glutamate residue and the coordinating water molecule, for which higher distances to metal were found in the C-terminal cupin domain. The metal geometry in the N-terminal cupin domain resembled the major (70%) trigonal bipyramidal arrangement found for free *A. japonicus* quercetinase while that in the C-terminal cupin domain was related to the minor (30%) square pyramidal arrangement. In order to get insight upon the role of the metal in quercetinase catalysis, overexpressed YxaG was demetalated (DDTC diethyldithiocarbamate, EDTA) then tentatively reconstituted with various metal ions (Ca<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Fe<sup>3+</sup>, Fe<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Cd<sup>2+</sup>). Enzymatic activities similar to the one of native enzyme were recovered only when Co<sup>2+</sup> (116%) and Cu<sup>2+</sup> (98%) were used. Activities of 59, 49, 35, 12 and 7% were obtained with Mn<sup>2+</sup>, Fe<sup>3+</sup>, Ni<sup>2+</sup>, Fe<sup>2+</sup> and Cd<sup>2+</sup> respectively, while no activity



**Fig. 14** The bi-metallated bicupin nature of *B. subtilis* quercetinase (PDB code 1y3t)

could be restored with Ca<sup>2+</sup>, Mg<sup>2+</sup> and Zn<sup>2+</sup> bringing into question the exact nature of the metallic cofactor.

#### What is the relevant metal in YxaG catalysis?

By carefully controlling the metal content of the medium used to overexpressed YxaG in *E. coli*, it was found that adding at the induction time Mn<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup> or Cu<sup>2+</sup> at a final concentration of 10 μM, resulted respectively in a 35-, 24-, 2.6- and 1.4-fold increase quercetinase activity as compared to no addition. Addition of Fe<sup>2+</sup>, Zn<sup>2+</sup> and Cd<sup>2+</sup> had no influence over control. Since the purification of native YxaG from *B. subtilis* is still awaiting, the nature of the metal content in it could not be known. Nevertheless the previous results suggested that this enzyme is a Mn-dioxygenase.

The Mn- and Co-YxaG were purified and biochemically characterised (Schaab et al. 2006). Various compounds used at 1 mM (kojic acid, H<sub>2</sub>O<sub>2</sub>, NaCN, *O*-ethyl xanthic acid, diethyldithiocarbamate, EDTA, DTT, sodium ascorbate and NaN<sub>3</sub>) were poorly inhibitory. The kinetic parameters for both Mn- and Co-YxaG were determined and compared to Fe-YxaG. The K<sub>m</sub> for quercetin was approximately the same for the three enzymes (ranging from 4 to 7.5 μM), the K<sub>m</sub> for dioxygen shown similar variations (ranging from 79 to 150 μM) but the k<sub>cat</sub> were largely different (25, 6.7 and 0.65 s<sup>-1</sup> for respectively Mn-, Co- and Fe-YxaG). A pH dependant study of K<sub>m</sub> and V<sub>max</sub>, shown that a single ionisable group participate in

catalysis. X-band EPR analysis of Mn-YxaG was consistent with an octahedral geometry with O- and N-ligands. Anaerobic addition of quercetin has poor effect on the spectrum. Finally, the fact that Mn was found in the reduced state ( $Mn^{2+}$ ) suggested that dioxygen and not quercetin was activated during catalysis.

#### Regulation of *yxaG* in response to quercetin

As recently shown (Yoshida et al. 2004; Hirooka et al. 2007), the *yxaG* gene is part of an operon *yxaGH*, itself included in the LmrA/YxaF regulon, further comprising the *yxaF* gene and the *lmrAB* operon. LmrA and YxaF are two transcriptional Tet-R type repressors that are paralogous to each other, the former acting on both *yxaGH* and *lmrAB* operons, the latter preferentially on the *yxaGH* operon. *LmrB* encodes a multidrug resistance pump, *yxaG* the quercetinase described above and *yxaH* a membrane protein of unknown function. Some flavonoids, among them quercetin, were able to inhibit the binding of both LmrA and YxaF to DNA, thus allowing the synthesis of LmrB, YxaG and YxaH. Indeed when *Bacillus subtilis* mutants with *yxaF*, *lmrA* or both disrupted genes were grown in a free quercetin medium, they expressed quercetinase activity while wild strain was virtually unable.

#### *Streptomyces* sp. quercetinase

In addition to bicupin quercetinases (moulds, *Bacillus subtilis*), a monocupin quercetinase has been recently described from a *Streptomyces* sp. strain (Merkens et al. 2007). This strain denoted FLA along with other *Streptomyces* (Merkens et al. 2007), was able to decolorize quercetin agar but when measured, the growth of FLA strain on quercetin supplemented mineral medium was extremely low, excluding quercetin as an efficient metabolic source of carbon and/or energy. Nevertheless quercetin was shown to induce intracellular quercetinase activity. From a four-step-purification procedure and starting from 38 g of wet biomass, 0.2 mg of purified quercetinase (noted QueD) were obtained with a specific activity of 98 U/mg. This allowed, after N-terminal sequence analysis, the cloning and sequencing of the coding gene *queD*. In a further study (Merkens and Fetzner 2008) the transcriptional analysis of *queD* was performed. Flavonols such as quercetin and rutin (but not morin) were implicated in the regulation of

*queD* gene, acting as inducers. Glucose, rhamnose as well as protocatechuate and phloroglucinol carboxylic acids had no influence on the process nor had  $Ni^{2+}$  ions, the preferred metallic cofactor of QueD (see below). Sequence analysis of DNA regions flanking *queD* gene suggested that the quercetinase gene from *Streptomyces* sp. FLA is not part of a catabolic gene cluster (Merkens et al. 2007) but part of an operon since two ORFs upstream of *queD* are co-transcribed with the latter (Merkens and Fetzner 2008). These two ORFs coded for a putative amidohydrolase and a putative dienelactone hydrolase or carboxylesterase, enzymes that might be involved in depside hydrolysis following quercetinase action on flavonols (Merkens et al. 2007; Merkens and Fetzner 2008).

As already mentioned QueD quercetinase showed two interesting features when compared with other quercetinases. First of all, *queD* gene is coding for a monocupin of 186 amino acids and 21,065 Da. The four canonical metal binding residues (H, H, E, H) were present within the two consensus sequence signature of cupins, suggesting that *Streptomyces* sp. FLA quercetinase was a metalloprotein. Sequence alignment showed that the monocupin *Streptomyces* quercetinase was more related to the C-terminal and the N-terminal cupin domains of *B. subtilis* (35.9% identity) and *A. japonicus* (27.9% identity) quercetinases respectively. The second interesting feature of QueD quercetinase was that  $Ni^{2+}$  looked to be the preferred metallic cofactor of the enzyme (Merkens and Fetzner 2008; Merkens et al. 2008) followed by cobalt. Indeed, when crude extract preparations of *Streptomyces* sp. FLA or *E. coli* heterologously expressing QueD His<sub>6</sub> grown in  $Ni^{2+}$  or  $Co^{2+}$  supplemented culture medium, were analysed for quercetinase specific activity, a 16 and 6.1 fold respective increase was noted for Ni over non supplemented culture media and a 7.6 and 1.6 fold respective increase was noted for Co over non supplemented culture media. However, when analyses for metal content were conducted, an approximate 0.5 equivalent of Ni or Co were detected for QueD His<sub>6</sub> respectively obtained from Ni and Co supplemented media (Merkens et al. 2008). Both enzymes were well folded and exhibited identical secondary structures (CD spectra) with a high content in  $\beta$ -sheet. In both cases carbon monoxide and the expected depside were analysed after quercetin transformation (Merkens et al. 2008). Purified recombinant Ni-QueD and Co-QueD

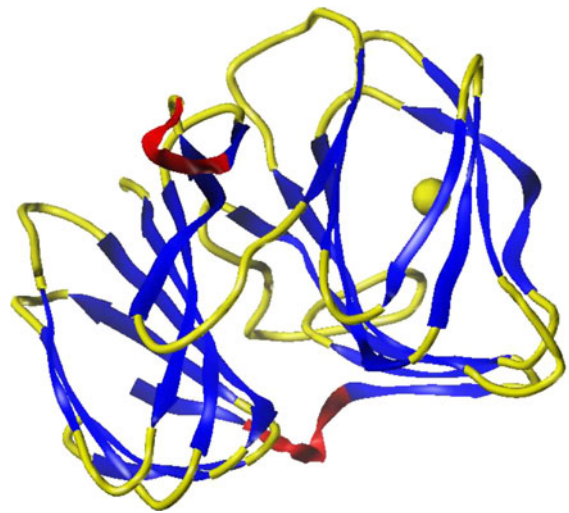
exhibited specific activity of 144 and 28 U/mg, respectively, both enzymes showing similar catalytic efficiency ( $k_{\text{cat}}/K_m$ ) with quercetin as substrate.

EPR experiments (Merkens et al. 2008) revealed that Ni-QueD was EPR silent, something indicative of a paramagnetic Ni species whatever the presence of dioxygen or quercetin. To the contrary, EPR analysis Co-QueD revealed a high spin ( $S = 3/2$ )  $\text{Co}^{2+}$  species independent of the presence or absence of dioxygen. Anaerobic addition of the substrate led to a modified spectrum but redox state of the cobalt ion remained unchanged. Based on these results and other considerations, it was concluded that the metal ion in *Streptomyces* sp. FLA had probably no redox role.

### Pirins

Pirins are highly conserved bicupins (found in mammals, plants, fungi and prokaryotes) and are believed to act as co-transcriptional factor. As part of a study aiming to compare human pirin and a prokaryotic counterpart, structural studies were undertaken on *E. coli* pirin. By homologous expression of the His-tagged *E. coli* pirin, the enzyme could be overproduced ( $\sim 200$  mg/l) and easily purified in one metal chelating affinity chromatographic step. The structure of the protein was then determined at 2.0 Å resolution (Fig. 15) through X-ray diffraction experiments (Adams and Jia 2005).

The space group was  $P3_221$  with one monomer per asymmetric unit. The structural arrangement, similar to the one of human pirin (Pang et al. 2004), was typical of a bicupin showing two face to face similar domains each composed of two antiparallel  $\beta$ -sheets forming a  $\beta$ -sandwich. The two domains were linked by a short single turn helix. Only the N-terminal domain possessed the canonical three His-one Glu set of cupin metal ligands. Due to the crystallisation medium composition, an atom of Cd was shown to occupy the metal site. It should be noted that human pirin is a N-terminal mono-iron bicupin and that the metal analysis of the recombinant *E. coli* protein was found to contain mainly Zn, Cu and Fe atoms in a 4.05/1.18/1 ratio (ICP-MS). No metal/monomer ratio was given. By structural comparison with other bicupins and by manual docking, a large pocket in the N-terminal domain situated next to the metal site was found that could accommodate quercetin.



**Fig. 15** The mono-metallated bicupin nature of *E. coli* pirin (PDB code 1tq5)

Furthermore the global folds of both human and *E. coli* pirins aligned quite well with *A. japonicus* quercetinase structure. These results prompted the authors to test a putative quercetinase activity of both pirins. A significant bleaching of the quercetin chromophore was observed after pirin addition and released CO could be detected as well. Classical quercetinase inhibitors, such as kojic acid, diethylthiocarbamate and 1,10-phenanthroline, proved to completely inhibit quercetinase activity at 50 nM concentration. All these results lead to the conclusion that human and *E. coli* pirins could act as quercetinase and that their role could be to control the intracellular concentration of quercetin, a potent inhibitor of various enzymes and transcriptional processes (Adams and Jia 2005). In a recent study (Neznanov et al. 2008) the quercetinase activity of pirin was proved to lower the potential inhibitory role of quercetin on poliovirus replication in human kidney cell lines thus establishing a first biological role linked to the quercetinase activity of pirin.

### Conclusion

The goal of this work was to compile and analyse in the most extensive way the literature in the field of the RCP. As the first papers dealing with microbial aerobic degradation of flavonols had been published in 1959 (Hattori and Noguchi 1959; Westlake et al.



1959), this review came out 50 years later to show the evolution of this very interesting multidisciplinary topic. After an early burst of interest between the very late fifties and the beginning of the seventies, that allow to establish the microbiology and the biochemistry of the RCP and to pave the way for further research, the beginning of this millennium had shown a renewed interest for quercetinases. Genomics and structural data in particular had highlighted the fact that all known quercetinases ranging from prokaryotic to eukaryotic enzymes belonged all to the cupin superfamily. But beside this apparent uniformity, recent results showed an astonishing variety between quercetinase of various origins. Indeed monoNi-monocupin, diMn-bicupin, monoCu-bicupin quercetinases and monoFe-bicupin pirin all catalysed the same 2,4-dioxygenolytic cleavage of flavonols to carbon monoxide and a depside. This diversity in quercetinases as well as unresolved questions such as mechanistic details, quercetinase evolution, physiological role and global distribution of this enzyme will probably led in the future to a better knowledge in the field. Therefore, the use of microorganisms bearing the RCP or isolated enzymes of the path, could be envisioned to remove toxic flavonols from vegetal wastes, for valorisation studies of naturally occurring flavonols either as raw carbon and energy sources or as starting material for fine chemical production of carboxylic acid phenolics such as protocatechuic or phloroglucinol carboxylic acids through the use of quercetinase and esterase for example. To date and to the best of our knowledge only one example of potential industrial use as been described and patented by Unilever in the context of RCP that is the use of quercetinase as additive in enzymatic bleach composition for washing powders (van der Heiden et al. 1998).

**Acknowledgments** This work was supported in part by a grant (no. 10659) from the “Ministère Délégué à l’Enseignement Supérieur et à la Recherche” to Sylvain Tranchimand.

## References

- Adams M, Jia Z (2005) Structural and biochemical analysis reveal pirins to possess quercetinase activity. *J Biol Chem* 280:28675–28682
- Agarwall G, Rajavel M, Gopal B, Srinivasan N (2009) Structure-based phylogeny as a diagnostic for functional characterization of proteins with a cupin fold. *PLoS ONE* 4:e5736
- Antonczak S, Fiorucci S, Golebiowski J, Cabrol-Bass D (2009) Theoretical investigations of the role played by quercetinase enzymes upon the flavonoids oxygenolysis mechanism. *Phys Chem Chem Phys* 11:1491–1501
- Armand-Fraysse D, Lebreton P (1969) Recherches physiologiques sur les champignons III. Transformation métabolique de la rutine par les champignons lignivores. *Bull Soc Chim Biol* 51:563–578
- Barney BM, Schaab MR, LoBrutto R, Francisco WA (2004) Evidence for a new metal in a known active site: purification and characterization of an iron-containing quercetin 2,3-dioxygenase from *Bacillus subtilis*. *Protein Expr Purif* 35:131–141
- Barz W (1971) Über den abbau aromatischer verbindungen durch *Fusarium oxysporum* Schlecht. *Arch Mikrobiol* 78:341–352
- Bowater L, Fairhurst SA, Just VJ, Bornemann S (2004) *Bacillus subtilis* YxaG is a novel Fe-containing quercetin 2,3-dioxygenase. *FEBS Lett* 557:45–48
- Braune A, Gutschow M, Engst W, Blaut M (2001) Degradation of quercetin and luteolin by *Eubacterium ramulus*. *Appl Environ Microbiol* 62:5558–5567
- Brown SB, Rajananda V, Holroyd JA, Evans EGV (1982) A study of the mechanism of quercetin oxygenation by <sup>18</sup>O labelling. *Biochem J* 205:239–244
- Child JJ, Simpson FJ, Westlake DWS (1963) Degradation of rutin by *Aspergillus flavus*. Factors affecting production of the enzyme system. *Can J Microbiol* 9:653–664
- Child JJ, Oka T, Simpson FJ, Krishnamurty HG (1971) Purification and properties of a phenol carboxylic acid esterase from *Aspergillus flavus*. *Can J Microbiol* 17:1455–1463
- Clissold PM, Ponting CP (2001) JmjC: cupin metalloenzyme-like domains in jumonji, hairless and phospholipase A<sub>2</sub>β. *Trends Biochem Sci* 26:7–9
- Das S, Rosazza JPN (2006) Microbial and enzymatic transformations of flavonoids. *J Nat Prod* 69:499–508
- Dunwell JM (1998) Cupins: a new superfamily of functionally diverse proteins that include germins and plant seed storage proteins. *Biotechnol Genet Eng* 15:1–32
- Dunwell JM, Gane PJ (1998) Microbial relatives of the seed storage proteins of higher plants: conservation of motifs in a functionally diverse superfamily of enzymes. *J Mol Evol* 46:147–154
- Dunwell JM, Khuri S, Gane PJ (2000) Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin superfamily. *Microbiol Mol Biol Rev* 64:153–179
- Dunwell JM, Culham A, Carter CE, Sosa-Aguirre CR, Goodenough PW (2001) Evolution of functional diversity in the cupin superfamily. *Trends Biochem Sci* 26:740–746
- Dunwell JM, Purvis A, Khuri S (2004) Cupins: the most functionally diverse protein superfamily? *Phytochemistry* 65:1–17
- Fiorucci S, Golebiowski J, Cabrol-Bass D, Antonczak S (2004) Oxygenolysis of flavonoid compounds: DFT description of the mechanism for quercetin. *ChemPhysChem* 5:1726–1733
- Fiorucci S, Golebiowski J, Cabrol-Bass D, Antonczak S (2006) Molecular simulations reveal a new entry site in quercetin 2,3-dioxygenase. A pathway for dioxygen. *Proteins* 64:845–850

- Fiorucci S, Golebiowski J, Cabrol-Bass D, Antonczak S (2007) Molecular simulations bring new insights into flavonoid/quercecinase interaction mode. *Proteins* 67:961–970
- Fittipaldi M, Steiner RA, Matsushita M, Dijkstra BW, Groenen EJJ, Huber M (2003) Single-crystal EPR study at 95 Hz of the type 2 copper site of the inhibitor-bound quercetin 2,3-dioxygenase. *Biophys J* 85:4047–4054
- Fusetti F, Schröter KH, Steiner RA, van Noort PI, Pijning T, Rozeboom HJ, Kalk KH, Egmond MR, Dijkstra BW (2002) Crystal structure of the copper-containing quercetin 2,3-dioxygenase from *Aspergillus japonicus*. *Structure* 10:259–268
- Gallego MV, Pinaga F, Ramon D, Valles S (2001) Purification and characterization of an  $\alpha$ -L-rhamnosidase from *Aspergillus terreus* of interest in wine making. *J Food Sci* 65:204–209
- Gopal B, Madan LL, Betz SF, Kossiakoff AA (2005) The crystal structure of a quercetin 2,3-dioxygenase from *Bacillus subtilis* suggests modulation of enzyme activity by a change in the metal ion at the active site(s). *Biochemistry* 44:193–201
- Haluk JP, Metche M (1970) Transformation microbiologique de la quercétine par *Aspergillus niger* Van Tieghem. *Bull Soc Chim Biol* 52:667–676
- Hattori S, Noguchi I (1959) Microbial degradation of rutin. *Nature* 184:1145–1146
- Hay GW, Westlake DWS, Simpson FJ (1961) Microbial decomposition of rutin. *Can J Microbiol* 7:921–931
- Hirooka K, Kunikane S, Matsuoka H, Yoshida K-I, Kunamoto K, Tojo S, Fujita Y (2007) Dual regulation of the *Bacillus subtilis* regulon comprising the *lmrAB* and *yxaGH* operons and *yxaF* gene by two transcriptional repressors, *LmrA* and *YxaF*, in response to flavonoids. *J Bacteriol* 189:5170–5182
- Hund H-K, Breuer J, Lingans F, Hüttermann J, Kappel R, Fetzner S (1999) Flavonol 2,4-dioxygenase from *Aspergillus niger* DSM 821, a type 2 CuII-containing glycoprotein. *Eur J Biochem* 263:871–878
- Iacazio G (2005) Increased quercetinase production by *Penicillium olsonii* using fractional factorial design. *Process Biochem* 40:379–384
- Kaizer J, Balogh-Hergovich E, Czaun M, Csay T, Speier G (2006) Redox and non-redox metal assisted model systems with relevance to flavonol and 3-hydroxyquinolin-4(1H)-one 2,4-dioxygenase. *Coord Chem Rev* 250:2222–2233
- Kooter IM, Steiner RA, Dijkstra BW, van Noort PI, Egmond MR, Huber M (2002) EPR characterization of the mononuclear Cu-containing *Aspergillus japonicus* quercetin 2,3-dioxygenase reveals dramatic changes upon anaerobic binding of substrates. *Eur J Biochem* 269:2971–2979
- Krishnamachari V, Levine LH, Paré PW (2002) Flavonoid oxidation by the radical generator AIBN: a unified mechanism for quercetin radical scavenging. *J Agric Food Chem* 50:4357–4363
- Krishnamurthy HG, Simpson FJ (1970) Degradation of rutin by *Aspergillus flavus*. Studies with oxygen 18 on the action of a dioxygenase on quercetin. *J Biol Chem* 245:1467–1471
- Kurosawa Y, Ikeda K, Igami F (1973) Alpha-L-rhamnosidase of the liver of *Turbo cornutus* and *Aspergillus niger*. *J Biochem* 73:31–37
- Mamma D, Kalogeris E, Hatzinikolaou DG, Lekanidou A, Kekos D, Macris BJ, Christakopoulos P (2004) Biochemical characterization of the multi-enzyme system produced by *Penicillium decumbens* grown on rutin. *Food Biotechnol* 18:1–18
- Manzanares P, de Graaf LH, Visser J (1997) Purification and characterization of an  $\alpha$ -L-rhamnosidase from *Aspergillus niger*. *FEMS Microbiol Lett* 157:279–283
- Manzanares P, Orejas M, Ibanez E, Valles S, Ramon D (2000) Purification and characterization of an  $\alpha$ -L-rhamnosidase from *Aspergillus nidulans*. *Lett Appl Microbiol* 31:198–202
- Manzanares P, van den Broeck HC, de Graaf LH, Visser J (2001) Purification and characterization of two different  $\alpha$ -L-rhamnosidases, RhaA and RhaB, from *Aspergillus aculeatus*. *Appl Environ Microbiol* 67:2230–2234
- Medina ML, Kiernan VA, Francisco WA (2004) Proteomic analysis of rutin-induced secreted proteins from *Aspergillus flavus*. *Fungal Genet Biol* 41:327–335
- Medina ML, Haynes PA, Breci L, Francisco WA (2005) Analysis of secreted proteins from *Aspergillus flavus*. *Proteomics* 5:3153–3161
- Merkens H, Fetzner S (2008) Transcriptional analysis of the *queD* gene coding for quercetinase of *Streptomyces* sp. FLA. *FEMS Microbiol Lett* 287:100–107
- Merkens H, Sielker S, Rose K, Fetzner S (2007) A new monocupin quercetinase of *Streptomyces* sp. FLA: identification and heterologous expression of the *queD* gene and activity of the recombinant enzyme towards different flavonols. *Arch Microbiol* 187:475–487
- Merkens H, Kappl R, Jakob RP, Schmid FX, Fetzner S (2008) Quercetinase QueD of *Streptomyces* sp. FLA, a monocupin dioxygenase with a preference for nickel and cobalt. *Biochemistry* 47:12185–12196
- Mills ENC, Jenkins J, Marigheto N, Belton PS, Gunning AP, Morris VJ (2002) Allergens of the cupin superfamily. *Biochem Soc Trans* 30:925–929
- Monti D, Pisevcova A, Kren V, Lama M, Riva S (2004) Generation of an  $\alpha$ -L-rhamnosidases library and its application for the selective derhamnosylation of natural products. *Biotechnol Bioeng* 87:763–771
- Narikawa T, Karaki Y, Shinoyama H, Fujii T (1998) Rutin degradation by culture filtrates from *Penicillia*. *Nippon Nogeik Kaishi* 72:473–479
- Narikawa T, Shinoyama H, Fujii T (2000) A  $\beta$ -rutosidase from *Penicillium rugulosum* IFO 7242 that is a peculiar flavonoid glycosidase. *Biosci Biotechnol Biochem* 64:1317–1319
- Neznanov N, Kondratova A, Chumakov KM, Neznanova L, Kondratov R, Banerjee AK, Gudkov AV (2008) Quercetinase pirin makes poliovirus replication resistant to flavonoid quercetin. *DNA Cell Biol* 27:191–198
- Noguchi I (1963) The degradation of flavonols by *Pullularia fermentans* var. *candida*. *Bot Mag Tokyo* 76:191–198
- Oka T, Simpson FJ (1971) Quercetinase: a dioxygenase containing copper. *Biochem Biophys Res Commun* 43:1–5
- Oka T, Simpson FJ (1972) Degradation of rutin by *Aspergillus flavus*. Quercetinase: isolation of a low molecular weight form containing less carbohydrate. *Can J Microbiol* 18:1171–1175
- Oka T, Simpson FJ, Child JJ, Mills SC (1971) Degradation of rutin by *Aspergillus flavus*. Purification of the dioxygenase, quercetinase. *Can J Microbiol* 17:111–118

- Oka T, Simpson FJ, Krishnamurthy HG (1972) Degradation of rutin by *Aspergillus flavus*. Studies on specificity, inhibition and possible reaction mechanism of quercetinase. *Can J Microbiol* 18:493–508
- Omori T, Shiozawa K, Sekiya M, Minoda Y (1986) Formation of 2,4,6-trihydroxy-carboxylic acid and 2-protocatechuoylphloroglucinol carboxylic acid from rutin by bacteria. *Agric Biol Chem Tokyo* 50:779–780
- Padrn J, Grist KL, Clark JB, Wender SH (1960) Specificity studies on an extracellular enzyme preparation obtained from quercetin grown cells of *Aspergillus*. *Biochem Biophys Res Commun* 3:412–416
- Pang H, Bartlam M, Zeng Q, Miyatake H, Hisano T, Miki K, Wong L, Gao GF, Rao Z (2004) Crystal structure of human pirin. *J Biol Chem* 279:1491–1498
- Pietta P-G (2000) Flavonoids as antioxidants. *J Nat Prod* 63:1035–1042
- Pillai BVS, Swarup S (2002) Elucidation of the flavonoid catabolism pathway in *Pseudomonas putida* PML2 by comparative metabolic profiling. *Appl Environ Microbiol* 68:143–151
- Puti M, Kalra S (2005) Purification and characterization of naringinase from a newly isolated strain of *Aspergillus niger* 1344 for the transformation of flavonoids. *World J Microbiol Biotechnol* 21:753–758
- Rajavel M, Kulkarni NN, Gopal B (2008) Conformational studies suggest that the double stranded  $\beta$  helix scaffold provides an optimal balance between protein stability and function. *Protein Pept Lett* 15:244–249
- Rao JR, Cooper JE (1994) Rhizobia catabolize *nod* gene-inducing flavonoids via C-ring fission mechanisms. *J Bacteriol* 176:5409–5413
- Rao KV, Weisner NT (1981) Microbial transformation of quercetin by *Bacillus cereus*. *Appl Environ Microbiol* 42:450–452
- Rao JR, Sharma ND, Hamilton JTG, Boyd DR, Cooper JE (1991) Biotransformation of the pentahydroxy flavone quercetin by *Rhizobium loti* and *Bradyrhizobium* stains (Lotus). *Appl Environ Microbiol* 57:1563–1565
- Rose K, Fetzner S (2006) Identification of linear plasmid pAM1 in the flavonoid degrading strain *Actinoplanes missouriensis*<sup>T</sup> (DSM 43046). *Plasmid* 55:249–254
- Schaab MR, Barney BM, Francisco WA (2006) Kinetic and spectroscopic studies on the quercetin 2,3-dioxygenase from *Bacillus subtilis*. *Biochemistry* 45:1009–1016
- Schneider H, Blaut M (2000) Anaerobic degradation of flavonoids by *Eubacterium ramulus*. *Arch Microbiol* 173:71–75
- Schoefer L, Mohan R, Schwiertz A, Braune A, Blaut M (2003) Anaerobic degradation of flavonoids by *Clostridium orbiscindens*. *Appl Environ Microbiol* 69:5849–5854
- Siegbahn PEM (2004) Hybrid DFT study of the mechanism of quercetin 2,3-dioxygenase. *Inorg Chem* 43:5944–5953
- Simpson FJ, Talbot G, Westlake DWS (1960) Production of carbon monoxide in the enzymatic degradation of rutin. *Biochem Biophys Res Commun* 2:15–18
- Simpson FJ, Narasimhachari N, Westlake DWS (1963) Degradation of rutin by *Aspergillus flavus*. The carbon monoxide producing system. *Can J Microbiol* 9:15–25
- Steiner RA, Kalk KH, Dijkstra BW (2002a) Anaerobic enzyme substrate structures provide insight into the reaction mechanism of the copper-dependent quercetin 2,3-dioxygenase. *Proc Natl Acad Sci USA* 99:16625–16630
- Steiner RA, Kooter IM, Dijkstra BW (2002b) Functional analysis of the copper-dependent quercetin 2,3-dioxygenase. 1. Ligand-induced coordination changes probed by X-ray crystallography: inhibition, ordering effect, and mechanistic insights. *Biochemistry* 41:7955–7962
- Steiner RA, Meyer-Klaucke W, Dijkstra BW (2002c) Functional analysis of the copper-dependent quercetin 2,3-dioxygenase. 2. X-ray absorption studies of native enzyme and anaerobic complexes with the substrates quercetin and myricetin. *Biochemistry* 41:7963–7968
- Tranchimand S, Tron T, Gaudin C, Iacazio G (2005) Evaluation of phenolics and sugars as inducers of quercetinase activity in *Penicillium olsonii*. *FEMS Microbiol Lett* 253:289–294
- Tranchimand S, Tron T, Gaudin C, Iacazio G (2006) First chemical synthesis of three natural depsides involved in flavonoid catabolism and related to quercetinase catalysis. *Synth Commun* 36:587–597
- Tranchimand S, Ertel G, Gaydou V, Gaudin C, Tron T, Iacazio G (2008) Biochemical and molecular characterization of a quercetinase from *Penicillium olsonii*. *Biochimie* 90:781–789
- van den Bosch M, Swart M, van Gunsteren WN, Canters GW (2004) Simulation of the substrate cavity dynamics of quercetinase. *J Mol Biol* 344:725–738
- van der Heiden M, Nondmann DH, van der Helm MJ, Verrips CT, Swarthoff T, Smits A (1998) WO1997EP07138 19971210
- Westlake DWS (1963) Microbial degradation of quercitrin. *Can J Microbiol* 9:211–220
- Westlake DWS, Simpson FJ (1961) Degradation of rutin by *Aspergillus flavus*. Factors affecting production of the enzyme system. *Can J Microbiol* 7:33–44
- Westlake DWS, Spencer JFT (1966) The utilisation of flavonoid compounds by yeast and yeast like fungi. *Can J Microbiol* 12:165–174
- Westlake DWS, Talbot G, Blakley ER, Simpson FJ (1959) Microbial decomposition of rutin. *Can J Microbiol* 5:621–629
- Westlake DWS, Roxburgh JM, Talbot G (1961) Microbial production of carbon monoxide from flavonoids. *Nature* 189:510–511
- Winter J, Moore LH, Dowell VR Jr, Bokkenheuser VD (1989) C-ring cleavage of flavonoids by human intestinal bacteria. *Appl Environ Microbiol* 55:1203–1208
- Yoshida K-I, Ohki Y-H, Murata M, Kinehara M, Matsuoka H, Satomura T, Ohki R, Kumano M, Yamane K, Kunamoto K, Fujita Y (2004) *Bacillus subtilis* LmrA is a repressor of the *lmrAB* and *yxzGH* operons: identification of its binding site and functional analysis of *lmrB* and *yxzGH*. *J Bacteriol* 186:5640–5648