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REVIEW

α-L-rhamnosidase: production, properties, and applications

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Abstract

α-L-rhamnosidase [EC 3.2.1.40] belongs to glycoside hydrolase (GH) families (GH13, GH78, and GH106 families) in the carbohydrate-active enzymes (CAZy) database, which specifically hydrolyzes the non-reducing end of α-L-rhamnose. According to the sites of catalytic hydrolysis, α-L-rhamnosidase can be divided into α-1, 2-rhamnosidase, α-1, 3-rhamnosidase, α-1, 4-rhamnosidase and α-1, 6-rhamnosidase. α-L-rhamnosidase is an important enzyme for various biotechnological applications, especially in food, beverage, and pharmaceutical industries. α-L-rhamnosidase has a wide range of sources and is commonly found in animals, plants, and microorganisms, and its microbial source includes a variety of bacteria, molds and yeasts (such as *Lactobacillus* sp., *Aspergillus* sp., *Pichia angusta* and *Saccharomyces cerevisiae*). In recent years, a series of advances have been achieved in various aspects of α-validates the above-described-rhamnosidase research. A number of α-L-rhamnosidases have been successfully recombinant expressed in prokaryotic systems as well as eukaryotic systems which involve *Pichia pastoris*, *Saccharomyces cerevisiae* and *Aspergillus niger*, and the catalytic properties of the recombinant enzymes have been improved by enzyme modification techniques. In this review, the sources and production methods, general and catalytic properties and biotechnological applications of α-L-rhamnosidase in different fields are summarized and discussed, concluding with the directions for further in-depth research on α-L-rhamnosidase.

Keywords α-L-Rhamnosidase · Microorganism sources · Catalytic properties · Applications

Introduction

α-L-rhamnosidase (EC 3.2.1.40) is a glycoside hydrolase that can hydrolyze terminal L-rhamnose of various natural glycosides, such as naringin, hesperidin, and rutin (Table 1). The enzyme is ubiquitous in nature and is found in animals, plants and microorganisms. Based on amino acid sequence similarity, α-L-rhamnosidases of microbial origin can be classified into GH13, GH78 and GH106 glycoside hydrolase families in the CAZy database. The analysis of the protein structure and reaction mechanism of different α-L-rhamnosidase species revealed that they have some differences: GH78 family α-L-rhamnosidase contains five structural domains and reacts through the substrate binding to the catalytic domain of the barrel structure of $(\alpha/\alpha)_6$; GH106 family α-L-rhamnosidase has a $(\beta/\alpha)_8$-barrel structure containing five structural domains; GH13 family α-L-rhamnosidase crystal structure has not yet been resolved, and its properties are highly similar to the sequence of amylase but cannot hydrolyze soluble starch (Cui et al. 2007; Terry et al. 2020; Ndeh et al. 2017; Liu et al. 2012a, b). The main production mode of α-L-rhamnosidase is microbial fermentation. However, the current production level of microbial α-L-rhamnosidase needs to be improved, and the α-L-rhamnosidase produced by wild-type strains has problems such as the need for inducers and the difficulty of isolation and purification in the later stage. Although companies have realized the industrial production of this enzyme, the high price of commercial enzymes severely restricts the wide application of α-L-rhamnosidase. Therefore, so many researchers have been involved in the construction and modification of high-yielding α-L-rhamnosidase engineered strains.

α-L-rhamnosidase is one of the important enzymes for various biotechnology applications, especially in the food and pharmaceutical industries. As an enzyme with a natural glycosidic bond hydrolysis function, α-L-Rhamnosidase

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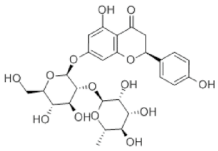
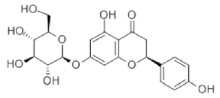
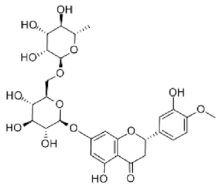
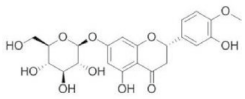
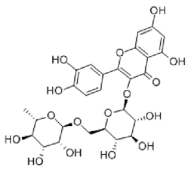
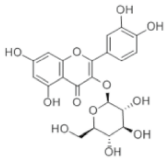
Table 1 α-L-rhamnosidase catalyzed hydrolysis of naringin, hesperidin, and rutin

Substrate			Product	
Chemical name	**Structural formula**	**Type of linkage**	**Chemical name**	**Structural formula**
naringin		α-1,2	prunin	
hesperidin		α-1,6	hesperetin 7-O-glucoside	
rutin		α-1,6	isoquercitrin	

acts to cleave the α-L-rhamnosyl portion of flavonoids to specifically hydrolyze the α-1,2, α-1,3, α-1,4, α-1, and α-1,6 glycosidic bonds at the ends of glycans or glycosides, releasing L-rhamnose and producing new glycans or glycosides (Slámová et al. 2018). For instance, it is used to remove bitter substances in citrus juice (Li et al. 2018b, 2019; Bodakowska-Boczniewicz and Garncarek 2019); improve the taste and aroma of fresh juice and tea (Fang et al. 2019; Peng et al. 2021a). Prunin, hesperetin 7-O-glucoside, and isoquercitrin with higher bioavailability and bioactivity are prepared using naringin, hesperidin and rutin as substrates (Carceller et al. 2019; Kumar et al. 2019; Wang et al. 2020). Although there have been review articles on α-L-rhamnosidase, many advances have been made in the research related to this enzyme in recent years. The purpose of this review is to summarize and discuss the progress in the sources, production, properties and applications of α-L-rhamnosidase so far, so as to further grasp the key research directions of α-L-rhamnosidase in the future.

Source and production

Animal and plant sources

Naringinase, which has both α-L-rhamnosidase and β-D-glucosidase activities, was first isolated from celery seeds by Hall (1938). Twenty years later, Ting (1958) and Thomas et al. (1958) also discovered naringinase in grape leaves. Since then some scholars have also studied naringinase in buckwheat seeds and the genus Rhamnus (Suzuki 1962; Bourbouze et al. 1975). Liver is the primary location of α-D-rhamnosidase in animals. Qian et al. (2005) first isolated and purified the α-L-rhamnosidase from pig liver, and they found that the enzyme could hydrolyze the terminal rhamnose of dioscin. Later, this dioscin-α-D-rhamnosidase was also identified and purified from bovine liver (Qian et al. 2013).

Microbial sources and production

Molds

The sources of mold include *Penicillium*, *Aspergillus*, and *Rhizopus* genera. By adding corn cob or naringin to the liquid medium, the researchers stimulated the formation of α-L-rhamnosidase. They then sequentially extracted and purified various α-L-rhamnosidase from *Penicillium citrinum* MTCC-3565, *Penicillium citrinum* MTCC-8897, and *Penicillium corylopholum* MTCC-2011 (Yadav et al. 2012a, b, 2013b). They also isolated and identified *Penicillium griseoroseum* MTCC-9224 from the decaying gooseberry fruit peel, which secretes α-L-rhamnosidase, and determined that gooseberry peel is the best inducer for its production of this enzyme (Yadav et al. 2017).

There are few reports on the production of α-L-rhamnosidase by *Rhizopus* species. Shanmugam and Yadav (1995) found that *Rhizopus nigricans* could produce α-L-rhamnosidase extracellularly. However, *Rhizopus arrhizus* CCF 100 could show obvious α-L-rhamnosidase activity only when it is induced by rutin or naringin (Monti et al. 2004).

The genus *Aspergillus* contains multiple known producers of α-L-rhamnosidase, including *Aspergillus terreus*, *Aspergillus aculeatus*, *Aspergillus niger*, *Aspergillus nidulans*, and *Aspergillus oryzae*. The production methods of α-L-rhamnosidase by *Aspergillus* mainly include liquid fermentation and solid fermentation. At present, the main international production mode is still liquid deep fermentation. Weignerova et al. (2012) fermented *A. terreus* CCF 3059 in a 75 L bioreactor to mass-produce alkali- and thermo-stable α-L-rhamnosidase. As with other *Aspergillus* spp., the α-L-rhamnocidase produced by *A. niger* tends to be more present as naringinase. Several investigations described the improvement of the fermentation medium and conditions for *A. niger* DB056 to produce naringinase, and after optimization, the maximum activity of α-L- rhamnosidase was 1069.30 U/mL in a 200 L fermenter (Wu et al. 2010).

Yeasts and other fungi

Pichia angusta X349 is regarded as one of the excellent producers of α-L-rhamnosidase (Yanai and Sato 2000). Furthermore, *Cryptococcus albidus* and *Candida tropicalis* can also produce α-L-rhamnosidase (Borzova et al. 2018). *Clavispora lusitaniae* has the capacity to produce α-L-rhamnosidase, according to Singh et al. (2015b). To improve the enzyme activity, the enzyme production conditions of this strain were further improved (Singh et al. 2015a).

Certain other fungi, in addition to molds and yeasts, can also produce α-L-rhamnosidase, e.g., the oat pathogenic fungus *Stagonospora avenae*, the soil fungus *Acrostalagmus luteoalbus*, and the plant pathogen *Fusarium moniliforme* (Bleddyn Hughes et al. 2004; Rojas et al. 2011; Kumar et al. 2019). A new α-L-rhamnosidase produced by *Talaromyces stollii* CLY-6 has been found to more easily hydrolyze the rhamnosidic linkage between the rhamnose and aglycone of epmedin C (Cheng et al. 2022).

Bacteria

Researchers have also focused a lot of emphasis on bacterial sources of α-L-rhamnosidase. The first bacteria found to produce α-L-rhamnosidase was *Bacteroides* JY-6, a strain of human intestinal bacteria (Jang and Kim 1996). Moreover, it has been demonstrated that the human gut bacteria *Fusobacterium* K-60 and *Enterococcus avium* EFEL009 secrete α-L-rhamnosidase (Park et al. 2005; Shin et al. 2016). Subsequent studies have revealed that some *Bacillus* bacteria, like *Bacillus litoralis* C44 and *Bacillus amyloliquefaciens* 11,568, are also able to produce α-L-rhamnosidase (Lyu et al. 2016; Zhu et al. 2017).

The abundance of marine and soil microorganisms has led to a continuous investigation of the productive properties of various bacteria in the ocean and soil by researchers. The bacterium *Pseudoalteromonas* sp. 005NJ, *Brevundimonas* sp. Ci19, and *Novosphingobium* sp. PP1Y screened from sub-Antarctic seawater, Beagle Channel, and contaminated seawater in Italy can produce α-L-rhamnosidase (Gastón Orrillo et al. 2007; Alvarenga et al. 2013; Izzo et al. 2014). As a fermentation strain, Rodrigues et al. (2020) used the *Acidobacterium bacterium* AB60, which was isolated from Cerrado soils. In the medium that contained xylan as a carbon source, they fermented it. In addition to the primary xylan-degrading enzymes, high enzyme activity α-L-rhamnosidase was present in the fermentation broth.

Molecular biology research of Microbial-derived α-L-rhamnosidase

Cloning of α-L-rhamnosidase genes

At the beginning of the 21st century, researchers have gradually started to study the cloning of α-L-rhamnosidase genes. Initially, PCR amplification and library construction were the most common methods to clone α-L-rhamnosidase genes from microorganisms. Recently, based on the continuous development and advancement of sequencing technology, metagenomic mining has also been applied to the study of α-L-rhamnosidase genes. The cloned α-L-rhamnosidase genes in representative microorganisms are listed in Table 2. Numerous research has revealed that the fungal and bacterial genome may contain multiple genes encoding α-L-rhamnosidase. For instance, the gene sequence of a novel rutin-converting flavonoid glycoside hydrolase was cloned from *A. niger* DLFCC-90 (accession number EU200666), and the enzyme was classified in the GH 13 family by sequence similarity comparison and hydrolysis characterization (Liu et al. 2012a, b).

Expression of α-L-rhamnosidase genes

So far, the expression of α-L-rhamnosidase of fungal and bacterial origin has been studied by many scholars. The expression of α-L-rhamnosidase from microorganisms in the last decade is demonstrated in Table 3. The prokaryotic (*E. coli*) expression system has been chosen for protein expression of the bacterial-derived α-L-rhamnosidase genes, and the pET system has been largely utilized. In *E. coli* BL21, the bacterial-derived α-L-rhamnosidase genes are expressed

Table 2 The cloned α-L-rhamnosidase genes in representative microorganisms

Organism	Name	pH optima	Temperature optima (°C)	Molecular weight	Accession number	References
Bacteria						
Clostridium stercorarium	*ramA*	7.5	60	95,000	AJ238748	Zverlov et al. (2000)
Bacillus sp. GL1	*rhaA*	6.5–7.0	40	98,280	AB046705	Hashimoto et al. (2003)
	rhaB	6.5–7.0	40	106,049	AB046706	
Sphingomonas paucimobilis FP2001	*rhaM*	–	–	97,400	AB080801	Miyata et al. (2005)
Lactobacillus plantarum NCC245	*rhaB1/rhaB2*	5	60	73,000	FJ943501	Avila et al. (2009)
Lactobacillus acidophilus DSM9126	$ramA_{LA}$	4	41	–	NC006814	Beekwilder et al. (2009)
Pediococcus acidilactici DSM 20,284	*ram*	5.5	50	76,800	ZP_07367044	Michlmayr et al. (2011)
	ram2	4.5	70	61,300	ZP_07366943	
Streptomyces avermitilis NBRC14893	*sav_828*	6	50	–	BAC68538	Ichinose et al. (2013)
Klebsiella oxytoca KCTC 1686	KoRha	5	–	–	YP_005019950	O'Neill et al. (2015)
Bifidobacterium dentium	BdRham	–	–	–	KF147170	Bang et al. (2015)
Bifidobacterium breve ATCC 15,700	–	6.5	55	87,000	CP006715.1	Zhang et al. (2015)
Novosphingobium sp. PP1Y	rRHA-P	6.9	45	120,000	WP_013837086.1	De Lise et al. (2016)
Bacteroides thetaiotaomicron VPI-5482	BtRha	6.5	55	83,300	WP_011107561.1	Wu et al. (2018)
Chloroflexus aurantiacus DSM636	–	6.0	50	105,000	A9WDK5	Shin et al. (2019)
Dictyoglomus thermophilum DSM 3960	*dicth_0289*	5	95	106,000	–	Guillotin et al. (2019)
Thermotoga petrophila DSM 13,995	*tpet_1682*	4.5	90	101,700	CP000702.1	Xie et al. (2020)
Fungi						
Aspergillus aculeatus	*rhaA*	5	40	92,000	AF284761	Manzanares et al. (2001)
	rhaB	5	40	85,000	AF284762	
Xylaria polymorpha	*gh78-1*	6		98,000	JN815084	Nghi do et al. (2012)
Aspergillus terreus	–				JN899401	Gerstorferová et al. (2012)
Aspergillus terreus	–				AFH54529	Spohner et al. (2015)
Alternaria sp. L1	*rhaL1*	6	60	–	JN704640	Xu et al. (2016)
Aspergillus oryzae RIB40	*AorhaA*	5	70	70,000	83,768,215	Ishikawa et al. (2017)
Aspergillus niger JMU-TS528	r-Rha1	5	60	–	AGN92963.1	Li et al. (2018b)
Aspergillus niger CCTCC M 2,018,240	*rha*	–	–	100,000	MH779610	Wang et al. (2019)
Aspergillus nidulans	*rhaE*	4.5	55	95,000	FR873475.1	Lyu et al. (2019)
Aspergillus tubingensis	AT-rRha	4	60	–	KX664478	Li et al. (2019)
Talaromyces stollii CLY-6	Rhase-TS	4.5	60	87,500	MT779018	Cheng et al. (2021)

stably with active protein (Wu et al. 2018; Ferreira-Lazarte et al. 2021).

The expression of α-L-rhamnosidase gene of *Aspergillus* origin has been more studied. Lyu et al. (2019) expressed the codon-optimized α-L-rhamnosidase gene from *n. E. coli* and obtained 574.5 U/L of *p*-nitrophenol-α-rhammnoside (*p*-NPR) hydrolase activity after fermentation in a 5 L bioreactor. Considering some problems inherent in the prokaryotic expression system, such as unstable disulfide bonds, incorrect protein folding, and inclusion body precipitation, the eukaryotic expression system is preferable for fungal-derived genes to avoid this set of possible problems. Spohner et al. (2015) cloned the codon-optimized gene encoding *Aspergillus terreus* rhamnosidase, and the

Table 3 Expression of α-L-rhamnosidase from microorganisms in the last decade

Organism	Expression vector	Expression host	Enzyme	Enzyme activity		References
				U/mL	U/mg	
Bifidobacterium dentium	pET-26b(+)	*E.coli* BL21(DE3)	BdRham	–	23.3	Bang et al. (2015)
Novosphingobium sp. PP1Y	pET-22b(+)	*E.coli* BL21(DE3)	RHA-P	–	5.9	Mensitieri et al. (2018)
Bacteroides thetaiotaomicron VPI-5482	pET-28a	*E.coli* BL21(DE3)	BtRha	–	0.57	Wu et al. (2018)
Thermotoga maritima MSB8	pET-24c(+)	*E.coli* BL21 Star	Tm_Ram106B	–	40.5	Baudrexl et al. (2019)
Chloroflexus aurantiacus DSM636	pET-28a(+)	*E.coli* BL21	–	0.6	304.3	Shin et al. (2019)
Thermotoga petrophila DSM 13,995	pET-28a	*E.coli* BL21(DE3)	TpeRha	–	105.03	Xie et al. (2020)
Lactobacillus plantarum WCFS1	pURI3-Cter	*E.coli* BL21(DE3)	Ram1	–	64.7	Ferreira-Lazarte et al. (2021)
Paenibacillus odorifer DSM 15,391	pET-20b	*E.coli* BL21(DE3)	PodoRha	–	49.3	Xie et al. (2022)
Dictyoglomus thermophilum	pRSFDuet-1	*E.coli* BL21(DE3)	DthRha	25.6	–	Yu et al. (2022)
Alternaria sp. L1	pPIC9K	*P.pastoris* GS115	RhaL1	2.27	–	Lu et al. (2015)
Aspergillus terreus	pKLacZ	*K.lactis* GG799	–	30.6	149.4	Spohner et al. (2015)
Aspergillus terreus CCF 3059	pPICZαA	*P.pastoris* KM71H (Mut^S)	A-Rha	–	82	Markosova et al. (2015)
Aspergillus oryzae RIB40	pPICZαC	*P.pastoris* GS115	AorhaA	0.36	5.4	Ishikawa et al. (2017)
Aspergillus nidulans	pET-28a(+)	*E.coli* BL21(DE3)	^{syn}AnRhaE	0.57	–	Lyu et al. (2019)
Aspergillus tubingensis	pPIC9K	*P.pastoris* GS115	AT-rRha	–	–	Li et al. (2019)
Aspergillus niger CCTCC M 2,018,240	pPIC9K	*P.pastoris* GS115	Rha	0.47	0.57	Wang et al. (2019)
Aspergillus niger	pCAMBIA	*A. niger* 3.350	Rha-N1	0.658	34.43	Ye et al. (2022)

hydrolase activity of the enzyme expressed in *Pichia pastoris* and *Kluyveromyces lactis* against *p*-NPR was 17.6 U/mL and 30.6 U/mL, respectively. Moreover, it is not difficult to find that most of the eukaryotic expression systems of *Aspergillus*-derived α-L-rhamnosidase genes are heterologously expressed using *Pichia pastoris*, rarely involve homologous expression. Only Ye et al. (2022) homologously expressed the α-L-rhamnosidase gene of *A. niger* in *A. niger* 3.350 and the *p*-NPR hydrolase activity of the target protein obtained after 5 L bioreactor fermentation was 34.43 U/mg.

Properties of α-L-rhamnosidase

General properties and catalytic properties of α-L-rhamnosidase

α-L-rhamnosidase from different sources tends to exhibit different properties. For future research and application of this enzyme, it is advantageous to have adequate knowledge of the properties of α-L-rhamnosidase from different sources. The general properties and catalytic properties of α-L-rhamnosidase from different microbial sources are summarized in Tables 4 and 5, respectively.

General properties of α-L-rhamnosidase

Different plants and animals and microorganisms differ in many aspects of their genetic composition, transcription, translation and post-translational modifications due to their species differences. Various sources of α-L-Rhamnosidase have different levels of glycosylation and different conformations of the enzyme protein due to different post-translational modification mechanisms, resulting in differences in their structure and properties. The enzymes of different sources also have different catalytic properties depending on the properties they carry with their production hosts. Most α-L-rhamnosidases have molecular weights between 50 and 140 kDa, with the majority falling in the around 100 kDa. The optimum pH, optimum temperature and thermal stability of enzymes are crucial for their industrial applications, so it is necessary to focus on these three properties of different α-L-rhamnosidases.

Three bacterial α-L-rhamnosidases have been found to have an acidic pH, despite the fact that the optimum pH range for most bacterial α-L-rhamnosidases is 5.0–8.0. Ram2 from *Pediococcus acidilactici* and TpeRha form *Thermotoga petrophila* both have an optimal pH of 4.5 (Michlmayr et al. 2011; Xie et al. 2020). The optimum

Table 4 General properties of α-L-rhamnosidase from different microbial sources

Organism	Molecular weight (kDa)	pH_{opt}	T_{opt} (℃)	Thermal stability	References
Pseudoalteromonas sp. 005NJ	–	6.0	40	Thermosensitive	Gastón Orrillo et al. (2007)
Lactobacillus acidophilus	–	6.0	37–45	Unstable	Beekwilder et al. (2009)
Streptomyces avermitilis	113	6.0	50	Stable below 40 °C	Ichinose et al. (2013)
Bifidobacterium breve	87	6.5	55	Stable at 60 °C	Zhang et al. (2015)
Novosphingobium sp. PP1Y	120	6.9	40.9	Stable between 25–40 °C	De Lise et al. (2016)
Bacillus amyloliquefaciens 11,568	32	7.5	45	Stable below 45 °C	Zhu et al. (2017)
Dictyoglomus thermophilum	100	5.0	95	Stable	Guillotin et al. (2019)
Thermotoga petrophila	101.7	4.5	90	Stable	Xie et al. (2020)
Paenibacillus odorifer	100	6.5	45	Stable at 40 °C	Xie et al. (2022)
Fusarium moniliforme MTCC-2088	36	10.5	50	Stable at 10 °C	Kumar et al. (2019)
Talaromyces stollii CLY-6	140	4.5	45	Stable below 50 °C	Cheng et al. (2022)
Aspergillus flavus MTCC-9606	41	11.0	50	Unstable	Yadav et al. (2011)
Aspergillus niger JMU-TS528	90	5.0	60	Stable	Li et al. (2016)
Penicillium griseoroseum MTCC-9224	97	6.5	57	Unstable	Yadav et al. (2017)
Aspergillus tubingensis JMU-TS529	110	4.0	50–60	Stable below 60 °C	Li et al. (2019)
Aspergillus nidulans	95	4.5	55	Stable below 55 °C	Lyu et al. (2019)
Aspergillus terreus CCF3059	130	6.5	65	Stable	Li et al. (2022)

Table 5 Catalytic properties of α-L-rhamnosidase from different microbial sources

Organism	*K*m (mM)	Substrate specificity	Links	References
Lactobacillus acidophilus	0.7	Naringin, rutin, hesperidin, narirutin	α-1,2, α-1,6	Beekwilder et al. (2009)
Streptomyces avermitilis	0.03	Naringin, rutin, hesperidin, gum arabic	α-1,2, α-1,6, α-1	Ichinose et al. (2013)
Bifidobacterium dentium	1.06	Naringin, poncirin, ginsenoside Re, rutin	α-1,2, α-1,6+	Bang et al. (2015)
Novosphingobium sp PP1Y	0.157	Naringin, rutin, hesperidin, quercitrin	α-1,2, α-1,6, α-1	Mensitieri et al. (2018)
Bacteroides thetaiotaomicron	2.87	Epimedin C, rutin, hesperidin	α-1,2, α-1,6	Wu et al. (2018)
Chloroflexus aurantiacus	–	Naringin, rutin, hesperidin	α-1,2, α-1,6+	Shin et al. (2019)
Dictyoglomus thermophilum	0.054	Naringin	α-1,2+, α-1,6	Guillotin et al. (2019)
Thermotoga petrophila DSM 13,995	2.99	Epimedin C	α-1,2	Xie et al. (2020)
Alternaria alternata SK37.001	4.84	Naringin, neohesperidin, rutin, hesperidin, quercitrin	α-1,2, α-1,6, α-1	Zhang et al. (2018)
Talaromyces stollii CLY-6	3.02	Epimedin C, naringin, neohesperidin, myricetrin, rutin, icariin	α-1,2, α-1,3, α-1,6, α-1	Cheng et al. (2022)
Papiliotrema laurentii ZJU-L07	1.38	Epimedin C, naringin, neohesperidin, rutin, hesperidin	α-1,2+, α-1,6	Lou et al. (2022)
Aspergillus awamori MTCC-2879	0.62	Naringin	α-1,2	Yadav et al. (2013a)
Aspergillus niger JMU-TS528	–	Naringin, myricetrin, saikosaponin C, rutin, hesperidin	α-1,2, α-1,3, α-1,4, α-1,6	Li et al. (2016)
Aspergillus oryzae NL-1	5.2	Naringin, rutin, hesperidin	α-1,2, α-1,6	Ge et al. (2017b)
Aspergillus nidulans	3.46	Epimedin C, naringin, neohesperidin, rutin	α-1,2+, α-1,6	Lyu et al. (2019)
Aspergillus terreus CCF3059	0.481	icariin	α-1	Li et al. (2022)

"*K*m" was detected using "*p*-NPR" as the substrate; + denotes that α-L-rhamnosidase has the strongest affinity for this glycosidic bond

pH of Ram2 from *Lactobacillus plantarum* WCFS1 is 3.0, which is the lowest optimum pH reported for acidic α-L-rhamnosidase (Ferreira-Lazarte et al. 2021). The optimum pH of fungal α-L-rhamnosidases is generally in the range of 5.0-6.5. Interestingly, some fungi can produce alkaline α-L-rhamnosidase. *Aspergillus clavato-nanicus* MTCC-9611, *Fusarium moniliforme* MTCC-2088, and *Aspergillus flavus* MTCC-9606 have the high optimum

pH of α-L-rhamnosidase, all between 10 and 11 (Yadav et al. 2011, 2012c; Kumar et al. 2019).

The optimum temperature for microbial α-L-rhamnosidase is typically in the range of 40–60 °C, but a few cold-tolerant and thermophilic enzymes do exist. The optimum temperature for α-L-rhamnosidase from the cold-tolerant bacterium, *Brevundimonas sp.* Ci19, is very low, at 20–37 °C (Alvarenga et al. 2013). Ram2 from *Pediococcus acidilactici*, RhaL1 from *Alternaria sp.* L1, and α-L-rhamnosidase from *Aspergillus terreus* CCF3059 all have an optimum temperature of 70 °C (Birgisson et al. 2004; Michlmayr et al. 2011; (Liu et al. 2012a, b; Weignerova et al. 2012). TpeRha from *Thermotoga petrophila* and DtRha from *Dictyoglomus thermophilum* both exhibit much higher optimum temperature than the above-mentioned thermophilic bacteria and fungi, at 90 and 95 °C, respectively (Guillotin et al. 2019; Xie et al. 2020). Moreover, TpeRha is the best heat-stable α-L-rhamnosidases to date, with the residual activity of more than 40% after 1 h incubation at 90 °C (Xie et al. 2020). According to some reports, the thermal stability of enzyme proteins is closely related to their degree of glycosylation modification, and to some extent, the glycosylation level of enzyme proteins correlates with their thermal stability (Manzanares et al. 2001). Besides, many other factors also affect the thermal stability of enzymes, such as salt bridges and hydrophobic interactions (Ge et al. 2018). Metal ions also have a significant effect on α-L-rhamnosidase activity. In the process of microbial metabolism or enzyme reaction in vitro, the participation of metal ions will affect the activity of enzyme. Mn^{2+} and Mg^{2+} can promote the conversion of naringin to prunin, while Cu^{2+} naringin can inhibit the conversion of prunin. The important role of Ca^{2+} in the catalytic process of α-L-rhamnosidase was revealed by comparing the crystal structures of the five available α-L-rhamnosidases. (Mensitieri et al. 2018) SaRha78A rhamnosidase, Ca^{2+} binds to an independent domain very close to the catalytic domain. When the substrate binds to the active site of the enzyme, Ca^{2+} can form coordination bonds with O_3 and O_4 of the substrate rhamnose-thus promoting the reaction (Fujimoto et al. 2013a, b). Enzyme activity inhibited by the metal chelating agent EDTA can also be fully restored by the addition of Ca^{2+}. Several experiments have demonstrated the positive effect of Ca^{2+} on glycosidase activity (Miake et al. 2000; Hashimoto et al. 1998). On the contrary, Hg^{2+} and sulfur-based reagents can affect the sulfur-based groups of enzymes, thus inhibiting the activity of some α-L-rhamnosidases (Yanai et al. 2000). When 1 mmol/L Hg^{2+} is present, the activity of BtRha and RamA and other glycosidases is significantly reduced or even completely inhibited (Zverlov et al. 2000; Wu et al. 2018).

Catalytic properties of α-L-rhamnosidase

α-L-rhamnosidase can act on a total of two types of glycosidic bonds, one is the bond between glycosyl group and aglycone directly, that is, α-1 glycosidic bond, and the other is the bond between glycosyl group and glycosyl group, including α-1,2, α-1,3, α-1,4, and α-1,6 glycosidic bonds. *P*-NPR is a synthetic substrate, and all α-L-rhamnosidases can act on its α-1 glycosidic bond except very few α-L-rhamnosidases. Therefore, *p*-NPR is a universal substrate for the determination of the enzyme activity of α-L-rhamnosidase. However, for quercetin, a natural glycoside containing the α-1 glycosidic bond, only a small fraction of α-L-rhamnosidase can hydrolyze it. For example, the α-L-rhamnosidases from *Streptomyces avermitilis*, *Novosphingobium* sp PP1Y, and *Alternaria alternata* SK37.001, while like most α-L-rhamnosidases, they can hydrolyze α-1,2 and α-1,6 glycosidic bonds (Ichinose et al. 2013; Mensitieri et al. 2018; Zhang et al. 2018). The specific hydrolysis is described by the process of hydrolysis of glycosidic bonds by rhamnosidase SaRha78A as an example. The amino acid Glu^{636} acts as a proton donor to attack the O1 of the substrate rhamnose to protonate it, while the amino acid Glu^{895} deprotonates the water molecules that have strong hydrogen bonds with the substrate and the enzyme, and the deprotonated water molecules attack the glycosidic bonds of the substrate to complete the hydrolysis reaction (Fujimoto et al. 2013a, b; David et al. 2000; Zhu et al. 2021).

Interestingly, different α-L-rhamnosidases may have higher specificity for one of the glycosidic bonds. For instance, the α-L-rhamnosidases from *Papiliotrema laurentii* ZJU-L07, *Dictyoglomus thermophilum*, and *A. nidulans* are more specific for the α-1,2 glycosidic bond (Guillotin et al. 2019; Lyu et al. 2019; Lou et al. 2022); whereas the α-L-rhamnosidases from *Chloroflexus aurantiacus* and *Bifidobacterium dentium* showed more specific hydrolysis of α-1,6 glycosidic bond (Bang et al. 2015; Shin et al. 2019). Moreover, some α-L-rhamnosidases can hydrolyze only one type of glycosidic bond exclusively, such as *A. tubingensis* JMU-TS529 whose α-L-rhamnosidase can only hydrolyze α-1,2 glycosidic bond (Li et al. 2019). The number of α-L-rhamnosidases that can hydrolyze α-1,3 and α-1,4 glycosidic bonds is small compared to α-1,2 and α-1,6 glycosidic bonds. Nevertheless, the α-L-rhamnosidase from *A. niger* S528 has a broad substrate spectrum, and it is the only α-L-rhamnosidase known to hydrolyze the above four glycosidic bonds (Li et al. 2016).

Epimedin C also contains the α-1,2 glycosidic bond, but unlike the glycosidic bonds linking rhamnose and glucose in other compounds, the glycosidic bond in epimedin C links two rhamnose groups. An increasing number of α-L-rhamnosidases have been shown to hydrolyze this α-1,2 glycosidic bond in epimedin C, including DthRha from

Dictyoglomus thermophilum DSM 3960, TpeRha from *Thermotoga petrophila* DSM 13,995, and Rhase-I from *Talaromyces stollii* CLY-6, and they further hydrolyze the α-1 glycosidic bond between rhamnose and aglycone in the product icariin (Xie et al. 2020; Zhang et al. 2021; Cheng et al. 2022).

Modification of α-L-rhamnosidase

Molecular modification of α-L-rhamnosidase

Thermal stability and catalytic efficiency are two important properties of enzymes, and studies aimed at improving these two properties have gradually increased in recent years. In particular, researchers have achieved many results in improving the thermal stability of r-Rha1 from *A. niger* JMU-TS528 through directed evolution and semi-rational design strategies (Li et al. 2018b; Liao et al. 2019). The most heat-stable r-Rha1 mutant available today, K406R/K573R, has a 3 h longer half-life at 60 °C than the wild type, which was obtained by replacing the two Lys on the surface of the enzyme with Arg (Li et al. 2018a). The affinity of r-Rha1 has been improved by the substitution of semi-conserved amino acids around the active site, and the affinity is the key to improve the catalytic efficiency (Li et al. 2020).

It should be emphasized that improving the catalytic efficiency needs to increase the flexibility of the structure, and improving the thermal stability needs to increase the rigidity of the structure, both of which have completely opposite structural requirements. Thus, it is difficult to improve the catalytic efficiency and thermal stability of α-L-rhamnosidase simultaneously by adjusting the protein structure. Li et al. (2021) proposed a dual screening strategy, which finally achieved the simultaneous enhancement of the thermal stability and catalytic efficiency of r-Rha1.

Immobilization of α-L-rhamnosidase

Immobilized enzymes can improve the thermal stability and affinity of the enzyme while maintaining the original properties of the enzyme, and at the same time make the enzyme easy to be separated and reusable. Metal-organic frameworks (MOFs), as an emerging porous material, have been successfully applied in the immobilization of α-L-rhamnosidase. For example, the α-L-rhamnosidase from *A. niger* CCTCC M 2,018,240 and *A. niger* JMU-TS528 were immobilized on magnetic MOFs and cerium-based metal-organic frameworks nanoparticles, respectively, and the substrate affinity of the enzymes were both significantly enhanced (Peng et al. 2021b; Wang et al. 2021). The co-immobilization of α-L-rhamnosidase Rha1 and β-glucosidase Glu4 from *Talaromyces stollii* CLY-6 was achieved based on a carrier-free cross-linked enzyme aggregate, and the co-immobilized enzyme exhibited more tolerant to sugars, thus becoming the enzyme that can obtain the highest icaritin yield at the highest epimedin C concentration ever reported (Liu et al. 2022).

Applications of α-L-rhamnosidase

Applications in the food industry

Citrus fruits have a bitter taste due to the presence of flavonoid glycosides such as hesperidin, neohesperidin, and naringin. Naringinase is often used for enzymatic debittering (Bodakowska-Boczniewicz and Garncarek 2019; Carceller et al. 2020). In fact, the presence of β-D-glucosidase is not required for debittering, but only the action of α-L-rhamnosidase can reduce the bitter substances. The fungus JMU-TS529 was isolated from rotten pomelo compost and identified as *A. tubingensis*, whose α-L-rhamnosidase has a strong hydrolytic effect only on naringin, and can remove the bitterness of pomelo juice while still retaining the aroma of pomelo (Li et al. 2019).

α-L-rhamnosidase alone or in combination with β-D-glucosidase can increase the floral flavor of orange juice, and the combined treatment has a more obvious effect on the taste and aroma quality of orange juice (Peng et al. 2021a). Ultrasonic action assisted α-L-rhamnosidase and β-D-glucosidase in the fermentation broth of *A. niger* to debitter Ouguan juice, and the rate of debittering increased while increasing the content of sweet and fruity aroma compounds in the juice, which improved the flavor of Ouguan juice (Gao et al. 2021). In addition to improving the aroma components of the juice, these two enzymes could also increase the content of aroma components in tea broth (Fang et al. 2019).

The hydrolysis products of α-L-rhamnosidase, flavonoid monoglycosides, have higher bioavailability and enhanced efficacy, so the beverage after the effect of α-L-rhamnosidase can be used as functional beverage, such as ginkgo tea drink (Fang et al. 2019). The addition of sorbitol promoted the hydrolysis of hesperidin by *A. niger* α-L-rhamnosidase, which helped to accelerate the production of hesperetin 7-O-glucoside, a sweetener precursor (Sun et al. 2022). The α-L-rhamnosidase immobilized on magnetic Fe3O4/MIL-101(Cr) nanoparticles could hydrolyze hesperidin dihydrochalcone to hesperidin dihydrochalcone glucoside, which is a sweetener (Wang et al. 2022).

Applications in the pharmaceutical industry

Rutin is converted to isoquercitrin by the action of α-L-rhamnosidase with the removal of one molecule of rhamnose. Isoquercitrin has anti-inflammatory, antioxidant, anti-allergic, and antihypertensive effects, and has important applications in the pharmaceutical industry. Yet, the

industrial production of isoquercitrin has problems such as high cost, slow reaction, and low yield. Ionic liquids as co-solvents, high hydrostatic pressure treatment, addition of sorbitol, and addition of organic solvents have all been used to address these issues (Wang et al. 2013; Kim et al. 2016; Ge et al. 2017a; Shin et al. 2019), but the conversion rate of rutin or the yield of isoquercitrin still could not reach the standard of industrial application. Wang et al. (2020) first hydrolyzed rutin to isoquercitrin using α-L-rhamnosidase in deep eutectic solvents. 130 g/L rutin could be completely converted to isoquercitrin, with the yield of isoquercetin reaching 208.68 mm/h.

Herba Epimedii is a famous Chinese herbal medicine. The total flavonoids of Epimedii (TFE) are the main active ingredients in Epimedii, including icariin, epimedin A, epimedin B, epimedin C and icariside II (Jiang et al. 2016). Among them, icariin has the most significant pharmacological activities, such as anti-osteoporosis, antidepressant, and treatment of cardiovascular diseases (Zhang et al. 2017). However, the content of icariin is limited, while epimedin C, which structurally has only one more rhamnose than icariin, has a higher content, so epimedin C can be used as a substrate to convert it into icariin using α-L-rhamnosidase, which can hydrolyze the α-1,2 glycosidic bond between two rhamnoses. *E.coli* BL21 cells expressing *A. nidulans* ^{syn}AnRhaE can completely convert 1 g/L of epimedin C to icariin within 90 min (Lyu et al. 2019). There are also many drug precursors or substances with multiple biological activities, such as tilianin and cyanidin-3-O-rutinoside, which can be prepared by α-L-rhamnosidase (Cui et al. 2016; Li et al. 2023).

L-rhamnose is used in the synthesis of rare bioactive rhamnosylated compounds in medicine and chemistry, and also functions as a chiral intermediate in plant protection agents (Yadav et al. 2010). All of the above mentioned substrates are subjected to α-L-rhamnosidase action to take off the terminal L-rhamnose, so α-L-rhamnosidase can be used to produce L-rhamnose (Wang et al. 2020). Moreover, some α-L-rhamnosidases can also synthesize glycosidic bonds through a reverse hydrolysis (Ge et al. 2017b). RhaL1 from *Alternaria* sp. L1 has been shown to perform rhamnosylation of anticancer drugs such as 2′-deoxy-5-fluorouridine, cytosine arabinoside, hydroxyurea, etc., and the rhamnosylated drugs are potentially valuable in enzyme-activated prodrug systems (Xu et al. 2019).

Conclusion

In recent years, many scholars have studied α-L-rhamnosidase from different levels. Firstly, the screening of α-L-rhamnosidase production strains and the optimization of fermentation conditions are becoming more and more mature, which provides a certain reference value for large-scale production of α-L-rhamnosidase preparations. Secondly, the cloning and expression of α-L-rhamnosidase from different microorganisms were studied at the molecular level. The constructed engineered strains with high enzyme activity can be used for producing α-L-rhamnosidase, which is of great significance for increasing the yield of this enzyme. Meanwhile, the properties of α-L-rhamnosidase from different microbial sources were studied, and the enzyme was modified by molecular modification or immobilization techniques, which is helpful to promote the practical application of α-L-rhamnosidase in industrial conditions. Finally, the catalytic ability of α-L-rhamnosidase to various natural substrates was studied, which provides ideas for the application of α-L-rhamnosidase in different production processes.

But so far, there are few studies on the induction mechanism and synthesis pathway of α-L-rhamnosidase from different sources. More in-depth studies on α-L-rhamnosidase should be carried out using structural biology and molecular biology techniques. Meanwhile, in order to better meet the demanding industrial production process, the enzyme modification work must continue to advance, and the search for more strategies that can simultaneously improve the catalytic efficiency and thermal stability of α-L-rhamnosidase should be taken as a research priority in the molecular modification of this enzyme. Furthermore, since the main applications of α-L-rhamnosidase include food and drug fields, α-L-rhamnosidase produced by *A. niger*, *A. oryzae*, and other filamentous fungi recognized as safe strains by the U.S. Food and Drug Administration, deserve the attention of researchers. In particular, the presence of β-D-glucosidase in the preparation of flavonoid monoglycosides catalyzed by α-L-rhamnosidase will cause the production of flavonoid aglycones as a by-product. It can be considered to use CRISPR/Cas9 gene editing technology to knockout the β-D-glucosidase gene, so that the complete transformation of flavonoid diglycosides to flavonoid monoglycosides can be achieved without the isolation and purification of α-L-rhamnosidase. The above research will continue to promote the deep understanding and further development of α-L-rhamnosidase in the future.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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