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A New α-Amylase from *Reticulitermes speratus* KMT1[1]

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ABSTRACT

Termites are wood pests that cause vast economic damage every year. They digest both cellulose and starch, but the enzymes for starch digestion have not been well characterized. We obtained complete amino acid sequence information on the KME1 α-amylase from *Reticulitermes speratus* KMT1 through analysis of total mRNA sequences. The KME1 enzyme has two α-amylase domains and is 68% identical to the α-amylase from *Blattellager manica*, its closest relative in the GenBank database. Some unique features of its conserved region and its distant evolutionary relationship to other insect α-amylases suggest that KME1 is a new type of α-amylase.

Keywords : Termite, *Reticulitermes speratus* KMT1, α-amylase, Homologous search, Phylogenetic analysis

1. INTRODUCTION

Termites efficiently degrade woody cellulose through a symbiotic collaboration with gut microorganisms, and they absorb the degradation products as nutrients (Nakashima *et al.*, 2002; Watanabe *et al.*, 1997; Zhou *et al.*, 2007). In the first step of cellulose degradation, termites secrete an endo-β-1,4-glucanase from their salivary glands that enzymatically reduces the size of cellulose. Later, symbiotic microorganisms in the termite gut secrete cellobiohydrolase and β-glucosidase to convert cellulose into glucose (Cho *et al.*, 2010; Nakashima *et al.*, 2002). Since starch is an energy-storing material in biological systems, termites should be able to use it as food. It has been shown that *Coptotermes formosanus* can survive on starch or glucose as a sole nutrient as well as on cellulose, and it does this without the assistance of symbiotic protozoans (Kanai *et al.*, 2008). However, only the sequence of a partial catalytic domain of the amylase gene from *Coptotermes formosanus* has so far been reported (GenBank Accession number: KC740998); no complete sequence is available.

Amylases are classified into three groups, α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2), and γ-amylase (EC 3.2.1.3), depending on where they cleave the α(1 → 4) glucosidic linkage on

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the target polysaccharides. Alpha-amylases cleave polysaccharides in a random manner and are the major amylase type in animals, including mammals. Although there is only 10% similarity between the amino acid sequences of α-amylases from animals, plants, and microbes, all α-amylases share four highly conserved domains (Nakajima *et al.*, 1986; Svensson *et al.*, 2002). Alpha-amylases from mammals, including humans, are mainly secreted from the salivary glands, and they help digest nutritional polysaccharides (Nater *et al.*, 2009). Alpha-amylases from plant pests also have a significant function in the digestive system (Franco *et al.*, 2002; Sharma *et al.*, 2010).

In this study, sequences of the entire isolated mRNA from the termite *Reticulitermes speratus* KMT1 were analyzed using next generation sequencing technology (unpublished data). Among the sequenced mRNAs, a new type of α-amylase gene was identified, and it has been reported here.

2. MATERIALS and METHODS

2.1. α-Amylase Coding Sequence Identification

Termites were collected from the Bukhan mountain in Seoul, Korea, and genetic analysis of mitochondrial cytochrome oxidase subunits I and II showed that these termites were *Reticulitermes speratus* KMT1 (Cho *et al.*, 2010). Total RNA of termites was purified using TRIzol reagent (Invitrogen, Life Technologies Korea Ltd. Seoul, Korea). Five hundred termites were mixed with 2 ml TRIzol reagent. The cells were homogenized with Tissue Lyser II (Qiagen Korea Ltd., Seoul, Korea). After rinsing with chloroform, total RNA was precipitated with isopropyl alcohol. The eukaryotic mRNA was purified using PolyATtract® mRNA Isolation Systems according to the manufacturer's manual (Promega Korea, Ltd., Seoul, Korea). The isolated mRNAs were sequenced using the GS-FLX system (Roche Diagnostics Korea Co., Ltd, Seoul, Korea). The sequence fragments were assembled using a GS De Novo Assembler (Roche Diagnostics Korea Co., Ltd). The functions of the assembled sequences were suggested by comparing the encoded proteins with homologous proteins found in a search of the protein database of the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). ORF finder (www.ncbi.nlm.nih.gov/projects/gorf/) was used to identify the longest open reading frame (ORF) similar to α-amylase. It was designated as α-amylase KME1 and registered in the NCBI GenBank database (accession number: KC477098).

2.2. Searching Conserved Domains and Homologous Proteins

The conserved domain of α-amylase KME1 was identified using a conserved domain database from NCBI (Marchler-Bauer *et al.*, 2011). Homologous proteins were identified by tblastn search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Among the top 500 identified proteins, the most homologous one from each family and two from the order Blattaria (to which *Reticulitermes speratus* belongs) were selected for further phylogenetic analysis.

2.3. Phylogenetic Analysis

Evolutionary relationships among the selected

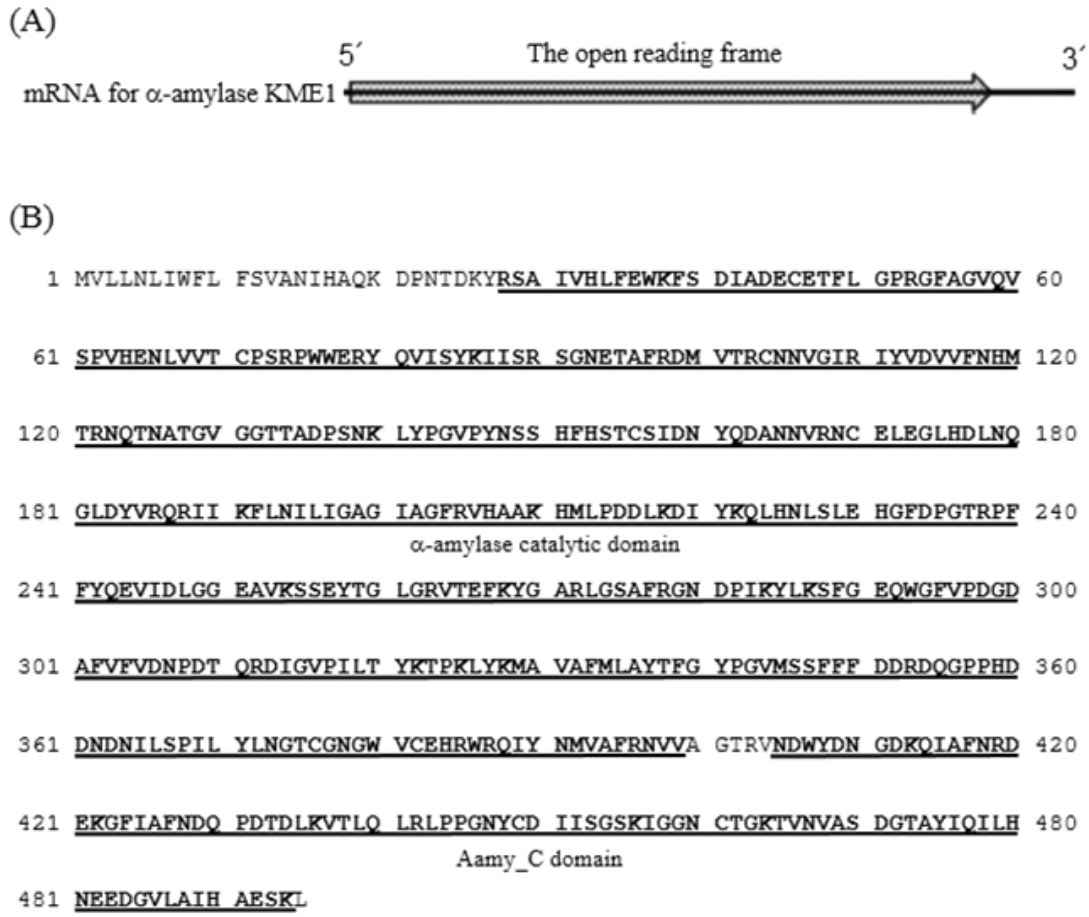

Fig. 1. Open reading frame (ORF) and protein sequence of α-amylase KME1. (A) The α-amylase KME1 ORF on the selected mRNA is indicated by an arrow. (B) The amino acid sequence of α-amylase KME1. The locations of the α-amylase catalytic domain and the Aamy_C domain are underlined and they are in bold characters.

22 proteins and α-amylase KME1 (NCBI protein accession #: AGJ52072) were analyzed using MEGA4 (Tamura *et al.*, 2007). All 23 protein sequences were aligned using ClustalW in MEGA4. Phylogenetic analysis was performed with the Bootstrap test with 1000 replicates using the neighbor-joining method.

3. RESULTS and DISCUSSION

3.1. The mRNA of α-amylase KME1

Total eukaryotic mRNA was purified from *Reticulitermes speratus* KMT1 and its sequence was analyzed using the GS-FLX system and GS De Novo Assembler (unpublished data). From the sequence data, the 1690-base mRNA for α-

Table 1. Selected proteins homologous with α-amylase KME1

Gene accession #	Protein accession #	Strain	Total score	Query coverage	E-value	Identity
KC477098	AGJ52072	*Reticulitermes speratus* (Termites)	1033	99%	0	100%
DQ355516	ABC68516	*Blattella germanica* (Cockroach)	686	96%	0	68%
AY945930	AAY23288	*Blattella germanica* (Cockroach)	667	99%	0	64%
HQ424576	ADP89000	*Hermetia illucens* (Fly)	604	97%	0	60%
AF146757	AAO13691	*Ceratitis capitata* (Fly)	600	99%	0	58%
XM_001975216	XP_001975252	*Drosophila erecta* (Fly)	580	99%	0	58%
XM_003494427	XP_003494475	*Bombus impatiens* (Bee)	579	97%	0	57%
XM_003704386	XP_003704434	*Megachile rotundata* (Bee)	576	99%	0	57%
X77318	CAA54524	*Litopenaeus vannamei* (Shrimp)	565	95%	0	58%
NM_001114376	NP_001107848	*Tribolium castaneum* (Beetle)	559	96%	0	57%
XM_312655	XP_312655	*Anopheles gambiae* (Mosquito)	556	96%	0	57%
AF208002	AAF20998	*Diabrotica virgifera* (Rootworm)	547	97%	0	56%
XM_001605971	XP_001606021	*Nasonia vitripennis* (Wasp)	546	95%	0	57%
HM357843	AEA76309	*Mamestra configurata* (Moth)	538	99%	0	53%
NM_001173153	NP_001166624	*Bombyx mori* (Silkworm)	531	99%	0	52%
FJ489868	ACL14798	*Ephestia kuehniella* (Moth)	528	96%	0	55%
NM_031502	NP_113690	*Rattus norvegicus* (Rat)	525	88%	2E-179	54%
AY330289	AAP92665	*Diatraea saccharalis* (Moth)	523	98%	1E-178	52%
XM_003505503	XP_003505551	*Cricetulus griseus* (Hamster)	521	99%	5E-178	54%
BC069347	AAH69347	*Homo sapiens* (Human)	513	99%	1E-174	52%
XM_003409529	XP_003409577	*Loxodonta africana* (Elephant)	512	98%	2E-174	53%
NM_001266186	NP_001253115	*Macaca mulatta* (Monkey)	511	98%	5E-174	53%
XM_003355277	XP_003355325	*Sus scrofa* (Boar)	509	99%	2E-173	53%

amylase was identified (data not shown). A poly(A) tail with 17 adenosines at the 3′ end indicates the eukaryotic origin of this mRNA. Open reading frame (ORF) analysis suggests the longest ORF starts at the 16th base with AUG and ends with UUG at the 1500th base followed by a UAA stop codon (Fig. 1A). The ORF of the mRNA sequence (1,485 bases) was submitted to the GenBank database of the NCBI (Accession number: KC477098), and the translated protein (495 amino acids) was assigned the accession number AGJ52072. A conserved domain search found an α-amylase catalytic domain from the 18th to the 399th amino acid and an α-amylase_C domain (Aamy_C domain in Fig. 1B) from the 405th to the 494th amino acid (Fig. 1B). Their E-values were 0 and 3.44e^{-24}, respectively. These results suggest an α-amylase function for AGJ52072, and the protein was therefore designated α-amylase KME1.

Protein	54	114	172	203	243	304	336	Strain
AGJ52072	GFAGVQVSP	DVVFN**H**	LEGLH**D**LN	GF**R**V**H**AAKH	Q**E**VIDLGGE	FVDN**PD**TQR--	AYTFGYPGVM	*Reticulitermes speratus*
AAY23288	GFAGVQVSP	DVILDH	LSGLHDLN	GLRVDAAKH	QEVIDQGGE	FVDNHDNQRGH	SHPYGYPRIM	*Blattella germanica*
ABC68516	GFAGVQVSP	DVVLNQ	LVGLHDLN	GFRVDAAKH	QEVIDLGGE	FVDNHDNQRGH	AYPYGYPRVM	*Blattella germanica*
XP_003505551	GFGGVQVSP	DAVINH	LSGLLDLA	GFRLDAAKH	QEVIDLGGE	FVDNHDNQRGH	AHPYGFTRVM	*Cricetulus griseus*
NP_113690	GFGGVQVSP	DAVINH	LSGLLDLA	GFRLDAAKH	QEVIDLGGE	FVDNHDNQRGH	AHPYGFTRVM	*Rattus norvegicus*
AAH69347	GFGGVQVSP	DAVINH	LSGLLDLA	GFRIDASKH	QEVIDLGGE	FVDNHDNQRGH	AHPYGFTRVM	*Homo sapiens*
NP_001253115	GFGGVQVSP	DAVINH	LTGLLDLA	GFRLDAASH	QEVIDLGGE	FVDNHDNQRGH	AHPYGFTRVM	*Macaca mulatta*
XP_003409577	GFGGVQVSP	DAVINH	LVGLVDLA	GFRIDASKH	QEVIDLGGE	FVDNHDNQRGH	AHPYGFTRVM	*Loxodonta africana*
XP_003355325	GFGGVQVSP	DAVINH	LVGLLDLA	GFRIDASKH	QEVIDLGGE	FVDNHDNQRGH	AHPYGFTRVM	*Sus scrofa*
CAA54524	GFAGVQVSP	DAVINH	LVGLNDLN	GFRIDASKH	QEVIDLGGE	FIDNHDNQRGH	AWPYGYTRVM	*Litopenaeus vannamei*
XP_312655	GYAGVQVSP	DLVINH	LVGLPDLN	GFRVDAVKH	QEVIDLGGE	FVDNHDNQRGH	AHPFGIPRIM	*Anopheles gambiae*
AAO13691	GFGGVQVSP	DVVFNH	LVGLKDLD	GFRVDAAKH	QEVIDLGGE	FVDNHDNQRGH	AHPFGITRVM	*Ceratitis capitata*
XP_001975252	GFAGVQVSP	DVVFNH	LVGLRDLN	GFRVDAAKH	QEVIDMGGE	FVDNHDNQRGH	AHPFGTPRVM	*Drosophila erecta*
ADP89000	GYGGVQVSP	DVVFNH	LVGLKDLD	GFRVDAAKH	QEVIDLGGE	FVDNHDNQR--	AHPYGITRIM	*Hermetia illucens*
ACL14798	GFGGIQISP	DAVINH	LVGLKDLD	GFRIDAAKH	HEVIDYGGE	FIDNHDNERGH	AHPYGEPQIM	*Ephestia kuehniella*
NP_001166624	GFGGIQVSP	DAIINH	LSGLKDLN	GFRIDAAKH	QEVIDLGGE	FIDNHDNQRGH	AHPYGYPQLM	*Bombyx mori*
AEA76309	GFGGIQISP	DAIINH	LSGLKDLN	GFRIDAAKH	QEVIDLGGE	FIDNHDNQRGH	AHPHGWPQLM	*Mamestra configurata*
AAP92665	GFGGIQISP	DAIINH	LSGLKDLN	GFRIDAAKH	QEVIDLGGE	FIDNHDNQRGH	AHPYGEPQLM	*Diatraea saccharalis*
XP_003494475	GYGGVQVSP	DAILNH	LTGLHDLD	GFRIDAAKH	QEVIDYGGE	FVDNHDTQRDN	AHPFGTPRVM	*Bombus impatiens*
XP_003704434	GFGGVQVSP	DAVVNH	LTGLHDLN	GFRIDAAKH	QEVIDYGGE	FVDNHDTQRDS	AHPFGTPRVM	*Megachile rotundata*
XP_001606021	GYAGVQVSP	DILLNH	LVGLHDLD	GFRVDAAKH	QEVIDYGGE	FIDNHDTQRSN	AHPYGIPRVM	*Nasonia vitripennis*
AAF20998	GFAGVQVSP	DVVINH	LTGLPDLD	GFRVDAAKH	QEVIDLGGE	FIDNHDNQRDG	AHPYGTTRIM	*Diabrotica virgifera*
NP_001107848	GFGGVQISP	DTVINH	LVGLADLN	GFRVDAAKH	QEVIDLGGE	FVDNHDNQRTG	AHPYGTTRLM	*Tribolium castaneum*
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	VI	I	V	II	III	IV	VII	

Fig. 2. Conserved domains on α-amylases, including KME1. Conserved domains identified by multiple sequence alignment of the proteins listed in Table 1. The conserved domains are numbered I–VII at the bottom of the Figure. The amino acids encoded by the conserved domains are emphasized by stars at the bottom of alignment. The α-amylase KME1 (AGJ52072) amino acids mentioned in the text are underlined and are in bold characters on its sequence.

3.2. Proteins Homologous to α–amylase KME1

Using the amino acid sequence of α-amylase KME1, the top 500 homologous proteins were identified from the NCBI GenBank database with a tblastn search. Most were α-amylases (data not shown). This result confirms that the AGJ-52072 protein is an α-amylase. Among the 500 homologous proteins, the most similar protein from each family and two proteins from the order Blattaria (to which *Reticulitermes speratus* belongs) were selected (Table 1). The most similar protein in the database was the α-amylase from *Blattella germanica*, a German cockroach, with a total score of 686, 96% query coverage, 0 E-value, and 68% identity. A low identity suggests that α-amylase KME1 is a new type of termite α-amylase. Because both *Reticulitermes speratus* and *Blattella germanica* are insects of the order Blattodea, it is reasonable that their α-amylases have the highest similarity. The *Blattella germanica* α-amylase (AAY23288) is known to be secreted from the tergal gland, but its biological function has not yet been determined experimentally (Saltzmann *et al.*, 2006).

Three flies (*Hermetia illucens*, *Ceratitis capitata*, and *Drosophila erecta*), two bees (*Bombus impatiens* and *Megachile rotundata*), one beetle (*Tribolium castaneum*), one mosquito (*Anopheles gambiae*), one rootworm (*Diabrotica virgifera*), one wasp (*Nasonia vitripennis*), three moths (*Mamestra configurata*, *Ephestia kuehniella*, and *Diatraea saccharalis*), and one silkworm (*Bomby*

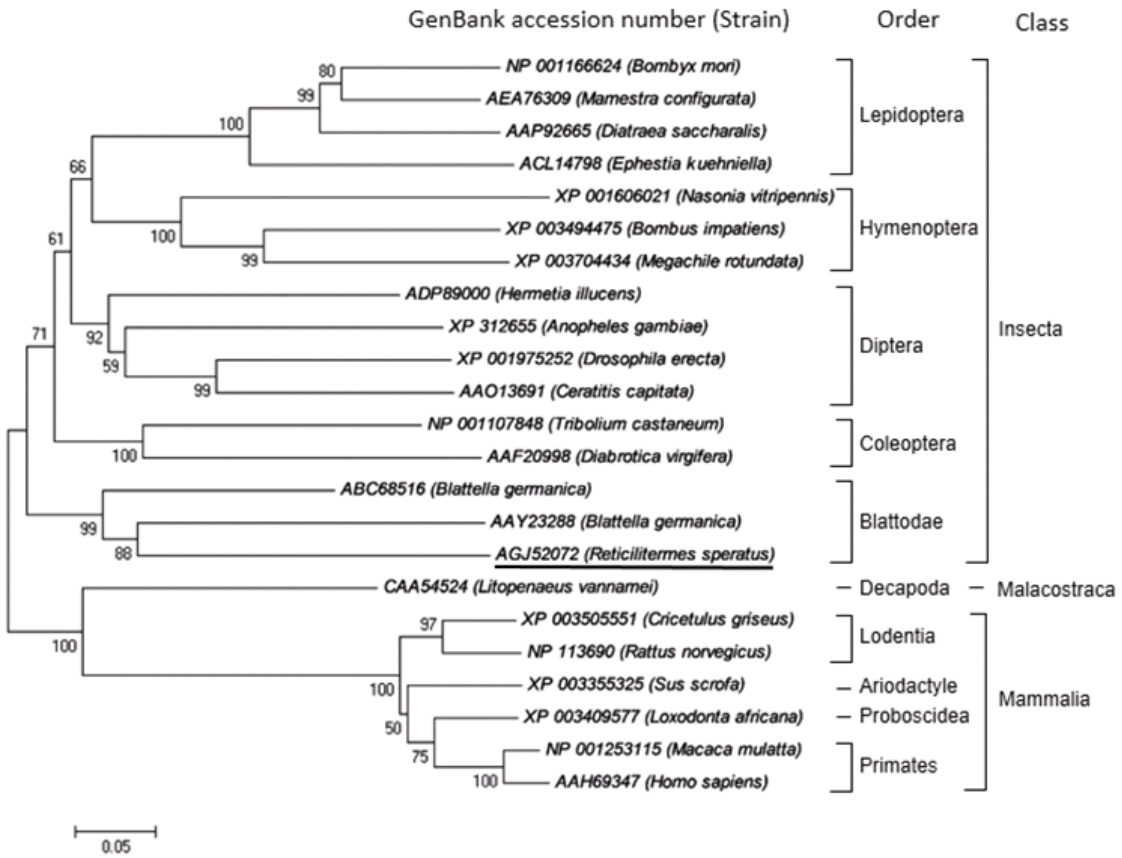

Fig. 3. Phylogenetic analysis of α-amylase KME1 (AGJ52072) with 22 selected proteins. GenBank accession numbers are used for protein identification. The order and the class of the hosts are indicated at the right side of the figure.

xmori) have α-amylases similar to that of *Reticulitermes speratus* KMT1. The wide distribution of α-amylases in the class Insecta suggests that they play an important metabolic role.

One shrimp (*Litopenaeus vannamei*) and six mammals (*Rattus norvegicus*, *Cricetulus griseus*, *Homo sapiens*, *Loxodonta africana*, *Macaca mulatta*, and *Sus scrofa*) are also listed in Table 1 as potential producers of α-amylases similar to α-amylase KME1.

3.3. Alignment of Amino Acid Sequences

The amino acid sequences of the KME1 α-amylase from *Reticulitermes speratus* KMT1 and 22 selected strains were aligned (Fig. 2). Seven highly homologous regions (I–VII; Fig. 2) found in α-amylases (Janeček, 2002; Janeč ek, 1997; MacGregor *et al.*, 2001; Nakajima *et al.*, 1986; Saltzmann *et al.*, 2006) are well conserved in all 23 α-amylases listed in Table 1. In the conserved regions, four amino acids are involved in the catalytic reaction and substrate

binding (Janeček, 2002). Alpha-amylase KME1 has three of the four, Glu^{244}, Asp^{309}, and Arg^{205}, but has His^{207} instead of Asp. In the case of Taka α-amylase A, two His amino acids were found to be important for its function even though they were not highly conserved in other related amylases (Janeček, 2002; Matsuura *et al*., 1984). Alpha-amylase KME1 has one of these histidines (His^{119}), but it has a proline (Pro^{308}) where the other His is located. Asp in conserved region V interacts with calcium ions (Janeček, 2002), which maintain the structural integrity of the active site (Janeček, 1997). Alpha-amylase KME1 also has Asp as the 177^{th} amino acid. Based on the comparisons of the conserved regions and amino acids, the KME1 α-amylase is a novel termite α-amylase.

3.4. Phylogenetic Tree

The evolutionary relationships among the 23 α-amylases in Table 1 were depicted using a phylogenetic analysis program (Fig. 3). First, α-amylases from the class Insecta were separated from those in the Malacostraca and Mammalia classes. Among Insecta α-amylases, those from the order Blattodea were separated first. The result of this analysis emphasized that Blattodea α-amylases, including KME1, are distinguishable from other α-amylases in the class Insecta. Termites are classified as belonging to the order Blattodea, whereas ants belong to Hymenoptera; thus, a high degree of similarity between the KME1 α-amylase and that from *Blattella germanica* is to be expected.

4. CONCLUSION

Although termites can survive with starch as the sole nutrient, their α-amylase digestive enzymes have not yet been well characterized. This study is the first to present complete protein sequence information on the α-amylase from *R. speratus* KMT1. Its low homology with the *Blattella germanica* α-amylase, some unique amino acids, and phylogenetic analysis all suggest that KME1 is a new type of α-amylase. Even the previously reported partial catalytic domain from *Coptotermes formosanus* is significantly different from the KME1 α-amylase. Further study is required to evaluate the significance of α-amylase KME1 and its contribution to the termite's metabolism.

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