

Variants of *Rhizomucor miehei* lipase and uses thereof

The present application claims priority to Singapore patent application number 10202103274U titled “Variants of *Rhizomucor miehei* lipase and uses thereof” filed on 30 March 2021 which
5 is incorporated by reference herein in its entirety.

Background

Triacylglycerol lipases belong to the class of hydrolases, which cleave the ester bond of carboxylic esters (E.C. 3.1.1.). The specific property of lipases is their ability to efficiently
10 cleave the ester bond between glycerol and fatty acids of lipids - different types of natural fats and oils. Currently, lipases are one of the most widely used class of enzymes with applications found in a wide range of industries including the detergent, pharmaceutical, food and biofuel industries.

15 *Rhizomucor miehei* lipase (RML) (mature peptide shown as SEQ ID No. 2) is one of the more commonly used lipases in industrial processes. One of its applications is in the production of human milk fat substitutes where it can perform transesterification or acidolysis. For different processes, the requirements for properties such as thermostability, chemical resistance, and pH for the optimal activity, as well as substrate specificity, of lipases differs. To suit the
20 applications of RML, engineering efforts have been made to improve its thermostability (Zhang et al., 2012) and enantioselectivity (Holmquist et al., 1993). However, regardless of application, specific activity remains as one of the most important properties of lipases since higher specific activity of enzyme allows savings in both enzyme and time. Thus, we have engineered variants of RML with improved activity.

25 Several ways are currently known for increasing specific activity of lipases. The most popular ways include manipulations of the substrate-binding pocket (Lafaquière et al., 2009), the lid domain (Khan et al., 2017) and the surface loops (Yedavalli et al., 2013). These manipulations can be achieved through random mutagenesis or site-directed mutagenesis. Previously, site
30 saturation mutagenesis had been successfully employed to identify RML variants with higher specific activity and greater stability for use in detergents (Balumuri et al., 2015).

Summary

In a first aspect of the invention, there is provided a lipase variant comprising a first substituent of a lipase sequence of SEQ ID No. 2, wherein a position of the first substituent in SEQ ID No. 2 is selected from the group consisting of position 251, position 204, position 254., position 237, and position 243. Preferably, the first substituent is less hydrophobic than phenylalanine.

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Preferably, the position of the first substituent is at position 251 and the first substituent is selected from the group consisting of asparagine, alanine, cysteine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, lysine, leucine, methionine, proline, glutamine, arginine, serine, threonine, valine, tryptophan and tyrosine. Advantageously, the phenylalanine at position 251 of the wild type may be substituted with any other natural amino acid.

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Preferably, the position of the first substituent is at position 204 and the first substituent is selected from the group consisting of more hydrophobic, less hydrophobic, polar and uncharged, negatively charged and positively charged, wherein the charge is determined with respect to the first substituent at a pH of 7. Preferably, the position of the first substituent is at position 204 and the first substituent is selected from the group consisting of phenylalanine, leucine, methionine, tyrosine, tryptophan, alanine, valine, glycine, methionine, proline, serine, threonine, asparagine, glutamine, aspartic acid, glutamic acid, arginine, histidine, and lysine. More preferably, the first substituent is selected from the group consisting of alanine, aspartic acid, phenylalanine, asparagine, and arginine. Advantageously, the isoleucine at position 254 of the wild type may be replaced by any natural amino acid.

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Preferably, the position of the first substituent is at position 254 and the first substituent is selected from the group consisting of less hydrophobic, polar, and negatively charged, wherein the charge is determined with respect to the first substituent at a pH of 7. Preferably, the position of the first substituent is at position 254 and the first substituent is selected from the group consisting of methionine, tyrosine, cysteine, alanine, glycine, serine, threonine, asparagine, glutamine, aspartic acid, and glutamic acid. More preferably, the first substituent is selected from the group consisting of alanine, aspartic acid, and asparagine.

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Preferably, the lipase variant comprises a second substituent of the lipase sequence of SEQ ID No.2, wherein the position of the second substituent in SEQ ID No. 2 is position 253, wherein the first substituent is preferably at position 251. Preferably, the second substituent is an amino acid with a polar uncharged side chain, for example threonine, asparagine, and glutamine,

preferably threonine. More preferably, the first substituent is at position 251 and selected from the group consisting of asparagine, alanine, aspartic acid, and glutamine.

Preferably, the lipase variant comprises a third substituent of the lipase sequence of SEQ ID No.2, wherein the position of the third substituent in SEQ ID No. 2 is position 156, wherein the first substituent is preferably at position 251. Preferably, the third substituent may be glycine. The D156X (preferably D156G) substitution may be paired with the F251X mutation (where X is any other amino acid) alone or in combination with F251X and S253X (preferably S253T) mutations.

Preferably, the first substituent is at position 251 and the lipase variant comprises a fourth substituent and a fifth substituent, wherein the fourth substituent and the fifth substituent is either at positions 237 and 239 respectively or positions 243 and 245 respectively. The fourth and fifth substituent may each independently be an amino acid with a polar uncharged side chain. For example, asparagine, threonine, serine, and glutamine as appropriate. In an example, the fourth substituent is asparagine, and the fifth substituent is threonine. Advantageously, the double substitution of asparagine and threonine (with an interposing amino acid, in other words 2 positions away) provide lipase variants with improved activity compared to the RML wild type.

Preferably, the lipase variant comprises a sequence selected from the group consisting of SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28, SEQ ID No. 29, SEQ ID No. 30, SEQ ID No. 31, SEQ ID No. 32, SEQ ID No. 33, SEQ ID No. 34, SEQ ID No. 35, SEQ ID No. 36, SEQ ID No. 37, SEQ ID No. 38, SEQ ID No. 39, SEQ ID No. 40, SEQ ID No. 41, SEQ ID No. 42, SEQ ID No. 43, SEQ ID No. 44, SEQ ID No. 45, SEQ ID No. 46, SEQ ID No. 47, SEQ ID No. 48, SEQ ID No. 49, SEQ ID No. 50, SEQ ID No. 51, SEQ ID No. 52, SEQ ID No. 53, SEQ ID No. 54, SEQ ID No. 55, SEQ ID No. 56, SEQ ID No. 57, SEQ ID No. 58, and SEQ ID No. 59.

Preferably, the lipase variant comprises a sixth substituent of a lipase sequence of SEQ ID No. 2, wherein the first substituent and the sixth substituent are at or proximal to a surface loop

region of the lipase sequence, wherein the first substituent is asparagine and the sixth substituent is threonine, the sixth substituent being two amino acid positions away from the first substituent. More preferably, the first substituent and sixth substituent are upstream of position 257.

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Preferably, the first substituent and sixth substituent is selected from the group consisting of S237N and L239T, D243N and S245T, and F251N and S253T.

For example, the lipase variant may comprise any one of SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, and SEQ ID No. 7.

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In various embodiments, the lipase variant has at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the sequence of SEQ ID NO: 2.

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In all aspects of the invention, the lipase variant comprises, or consists essentially of, or consists of, the substituent/s or SEQ ID No as described herein in the specification. For example, the lipase variant may comprise, or consists essentially of, or consists of, the first substituent of SEQ ID No. 2, and optionally additional substituents. For example, the lipase variant may comprise, or consists essentially of, or consists of, any one of the SEQ ID No. recited above.

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The charge of the amino acid side chain described herein is determined in water at pH 7 at 25 °C. The relative hydrophobicity of the amino acid side chain described herein is determined in water at pH 7, an example of a listing of the hydrophobicity of the natural amino acids is described in Monera *et al. J. Pept. Sci.*, 1995, 1, 319-329.

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In another aspect of the invention, there is provided a method comprising providing the lipase variant as disclosed herein or in the first aspect, a first ester and a reactant, wherein the reactant is selected from the group consisting of water, an acid and a second ester; and forming a reaction product between the first ester and the reactant with the aid of the lipase variant under suitable reaction conditions. The lipase variant may be used in different reactions including the hydrolysis of the first ester, and transesterification reactions with an acid or a different (i.e. a second) ester. More than one additional acid or ester as the reactant may be used. The

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transesterification reactions may be done in the absence of water or with minimal water present to avoid competing hydrolysis reactions. The lipase may catalyse the reaction between the reactants or via an intermediate which is not isolated.

- 5 The term “ester” as used herein includes fatty acid esters of glycerol like monoglyceride, diglyceride, triglyceride, and monoesters formed from short, medium, and long chain fatty acids and short chain alcohols like methanol, ethanol, propanol, and butanol including all structural isomers.
- 10 Preferably, the first ester is a fatty acid ester preferably a triacylglyceride. More preferably, the fatty acid ester or triacylglyceride is selected from the group consisting of olive oil, castor oil, sunflower oil, high oleic sunflower oil, rapeseed oil, high oleic rapeseed oil, palm oil, palm fraction enriched in tripalmitin, shea butter, shea olein, canola oil, glycerol trioctanoate, tributyrin, methyl octanoate, methyl decanoate, methyl dodecanoate, and vinyl laurate.

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- In an embodiment, the reactant is a medium chain fatty acid or ester. For example, the reactant is selected from the group consisting of octanoate (C8) ester, decanoate (C10) ester, dodecanoate (C12) ester, and mixtures thereof. The corresponding medium chain fatty acids may be used as well. Advantageously, this provides medium- and long-chain triacylglycerols (MLCT). For example, the reaction product may be a triacylglyceride with medium chain fatty acids at the 1,3-position of the triacylglyceride and a long chain fatty acid at the 2-position of the triacylglyceride. In other words, the reaction product has a formula of $R^1OCH_2CH(OR^2)CH_2OR^1$ wherein R^1 is a medium chain fatty acid and R^2 is a long chain fatty acid.
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In an embodiment, the reactant is a long chain fatty acid or ester. Examples include palmitic acid, stearic acid, oleic acid, linoleic acid and their corresponding esters.

- In an embodiment, the first ester is palm oil and the reactant is oleic acid, oleic ester, linoleic acid or linoleic ester. Preferably, the first ester is palm fraction enriched in tripalmitin (glycerol tripalmitate). Palm fraction refers to the isolation of a fraction of palm oil.
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In an embodiment, the fatty acid ester or triacylglyceride is selected from the group consisting of olive oil, high oleic sunflower oil, and high oleic rapeseed oil, and the reactant is stearic acid or stearic ester. The reaction product formed would be 1,3-distearoyl-2-oleoyl glycerol (SOS).

- 5 Preferably, the variant is SEQ ID No. 5. Preferably, water is provided as an aqueous buffer. Preferably, the variant is immobilized on a resin.

Preferably, the first ester and the reactant are in a liquid phase or a solution phase under the suitable reaction conditions. In other words, the first ester and reactant are miscible liquids or
10 dissolved in solution, for example a solvent is used to dissolve the first ester and reactant, or either the first ester or reactant acts as a solvent to dissolve the other.

Preferably, the reactant is the second ester, and the reaction product is a transesterification product of the first ester and the second ester. For example, one of the esters may be palm oil
15 or olive oil and the other ester may be at least one of a C8, C10, or C12 methyl ester, or any combinations thereof. In particular, it may be a mixture of C8, C10, and C12 methyl esters.

It will be appreciated that the MLCT product and SOS product (and other products formed by the methods herein) may be the major product formed but is unlikely to be the only product
20 formed.

In another aspect of the invention, there is provided a product obtained from the methods described above.

25 **Detailed Description of Embodiments of the Invention**

In the Figures:

Figure 1 shows the ribbon diagram of RML (PDB ID-3TGL; Brzozowski et al., 1993; PDB ID-6QPP; Moroz et al., 2019; Sehnal et al., 2018). Arrows indicate the positions of the amino acids 237 and 239, 243 and 245, and 251 and 253 which are mutated for (a) RML 237, (b) RML 243
30 (b) and (c) RML 251 respectively. Panel (d) of Figure 1 shows the position of amino acid 251 relative to the non-covalently bound propeptide.

Figure 2 shows the SDS-PAGE analysis of secreted protein in *P. Pichia* with heterologous expression of RML. The gel was stained with Coomassie blue for visualization. M, marker; WT, wild-type.

Figure 3 shows the specific activity of RML variants at various pH. Olive oil was used as substrate for the lipase activity assays at a pH of between 6 to 9. The values are expressed as mean $\pm$ SD ($n=3$).

Figure 4 shows 1,3-Dioleoyl-2-palmitoylglycerol (OPO) production by acidolysis using RML.

5 OPO content (% w/w) in reaction mixture was measured during the first four hours of incubation with immobilized RML WT and RML 251. The values are expressed as mean $\pm$ SD ($n=3$).

Figure 5 shows medium and long chain triacylglycerol (MLCT) production by transesterification using RML and RML251 (SEQ ID No. 5). C32 to C46 content (% w/w) in

10 reaction mixture was measured for the first six hours of incubation with immobilized RML WT and RML 251. The values are expressed as mean $\pm$ SD ($n=3$).

Features that are described in the context of an embodiment may correspondingly be applicable to the same or similar features in the other embodiments. Features that are described in the context of an embodiment may correspondingly be applicable to the other embodiments, even if not explicitly described in these other embodiments. Furthermore, additions and/or combinations and/or alternatives as described for a feature in the context of an embodiment may correspondingly be applicable to the same or similar feature in the other embodiments.

20 The following detailed description is merely exemplary in nature and is not intended to limit the invention or the application and uses of the invention. Furthermore, there is no intention to be bound by any theory presented in the preceding background of the invention or the following detailed description.

25 Other technical advantages may become readily apparent to one of ordinary skill in the art after review of the following figures and description.

Various amino acids are described herein by its full name, and conventional 1-letter and 3-letter abbreviations as is known in the art. The substitution of an amino acid residue in a peptide is describe by the conventional notation, for example F251N indicates that the 251th amino acid residue (or position) of phenylalanine (F) is substituted by asparagine (N).

As used herein, the articles “a”, “an” and “the” as used with regard to a feature or element include a reference to one or more of the features or elements. As used herein, the term “and/or”

includes any and all combinations of one or more of the associated listed items. As used herein, the terms “first,” “second,” and “third,” etc. are used merely as labels, and are not intended to impose numerical requirements on their objects. For example, the lipase variant may comprise the first substituent, and the third substituent without having the second substituent. In another
5 example, the lipase variant may comprise the first substituent, and the sixth substituent without having the second substituent, third substituent, fourth substituent, and fifth substituent. Other examples of different substituents are also possible. Thus, the terms “first,” “second,” and “third,” etc do not impose any numerical requirement.

10 The terms “polypeptide” and “protein”, used interchangeably herein, refer to a polymer of amino acids without regard to the length of the polymer; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not specify or exclude chemical or post-expression modifications of the polypeptides of the invention, although chemical or post-expression modifications of these polypeptides may be included or
15 excluded as specific embodiments. Therefore, for example, modifications to polypeptides that include the covalent attachment of glycosyl groups, acetyl groups, phosphate groups, lipid groups and the like are expressly encompassed by the term polypeptide. Further, polypeptides with these modifications may be specified as individual species to be included or excluded from the present invention. The natural or other chemical modifications, such as those listed in
20 examples above can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or
25 without branching. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of
30 pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. Also included within the definition are polypeptides which contain one or more analogs of an amino

acid (including, for example, non-naturally occurring amino acids, amino acids which only occur naturally in an unrelated biological system, modified amino acids from mammalian systems, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

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The terms “sequence similarity”, “percentage of sequence identity” and “percentage homology” are used interchangeably herein to refer to comparisons among polynucleotides and polypeptides, and are determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Identity is evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA, CLUSTAL W, FASTDB.

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The surface loop (positions 236-243 and 250 to 253 of SEQ ID No. 2) in the vicinity of the substrate binding pocket is modified by introduction of a N-glycosylation site (asparagine) to generate variants with increased hydrolytic activity between 34% and 115%. Additionally, variants were made with substituted amino acids at a region that could potentially interact with the propeptide. Structural studies of RML have suggested that, despite cleavage of the propeptide after protein maturation, it can remain in contact with the mature protein at the lid region to inhibit its activity (Moroz et al., 2019). Modifications of the amino acid at regions of contact could potentially dislodge the non-covalently attached propeptide. It may be possible that the sidechain of the substituted amino acids in RML may disrupt the interactions with the propeptide leading to improved activity of the modified RML. Through this method, improved hydrolytic activity towards olive oil by up to 153% has been obtained. The variants also demonstrated varying degrees of improvement in hydrolysis towards a wide range of substrates including castor oil, glycerol trioctanoate, tributyrin, C12 methyl ester, C8/C10 methyl esters and vinyl laurate.

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Examples

Site directed mutagenesis was performed to introduce mutations at amino acids in the vicinity of the substrate binding pocket (at or proximal to the surface loop region), in particular a pair of mutations comprising asparagine and threonine with an unmodified amino acid in between. The asparagine modification provides an N-glycosylation site and the threonine may promote the glycosylation. The following substitutions S237N + L239T, D243N + S245T and F251N + S253T were introduced and identified as variants RML237, RML243 and RML251, respectively (Figure 1). Compared to WT RML, the hydrolytic activities of RML237, RML243 and RML251 towards olive oil improved by 34%, 63% and 168%, respectively (results shown in Table 1). When the substrate was changed to castor oil, an improvement of 58% was also seen in RML237 and an increase of 253% was achieved by RML251 (Table 1). The hydrolysis of C8-C10 methyl esters was slightly improved by 20% with RML 237 and further increased by 96% with RML 251 but RML 243 showed decreased hydrolytic activity compared to the wild type RML.

Table 1: Relative hydrolytic activity of RML variants on olive oil and castor oil. The values are expressed as mean of triplicates.

	Relative specific activity (%)		
	Olive oil	Castor Oil	C8/C10 methyl esters
RML WT	100	100	100
RML 237	134	158	120
RML 243	163	84	12
RML 251	268	353	196
RML 237+251	224	-	-
RML 243+251	118	-	-

In terms of optimal temperature, a shift was observed amongst the variants as well. Instead of having an optimal temperature for hydrolysis at 55°C like for WT, RML237 and RML 251 achieved its highest activity at 50°C, while RML 243 has lowest optimal temperature of 40°C. These variants which require lower temperatures for optimal activity could help minimise the need for heating and thus save on energy costs. The optimal pH for activity remained at 8 for the variants and the activity at various pH are shown in Figure 4.

Variant with combined mutations RML 237+251 demonstrated an increase of 124% in specific activity compared to the wild type RML (WT). RML 243+251 was 18% more active than WT (Table 1).

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RML 251 was immobilized and used for acidolysis to produce 1,3-Dioleoyl-2-palmitoylglycerol (OPO), the amount of OPO accumulated was 51.8% more than RML WT after the first 30 min of reaction and 75.9% and 41.6% higher than RML at the end of 1h and 4 h respectively (Figure 4). The immobilized RML 251 was also used for transesterification to produce medium and long chain triacylglycerol (MLCT). At the end of 1h reaction, the proportion of C32-C46 accumulated was 20.7% higher for RML 251 than WT and continued to be slightly higher than WT at around 6% after 4h and 6h (Figure 5).

By replacing the amino acid at position 251 to other less hydrophobic amino acid or hydrophilic amino acids, the variants still had activities that are comparable to RML 251. For variants with double mutation of F251A/D/Q + S253T, their specific activities were 98 to 128% higher than WT for olive oil hydrolysis (Table 2). RML F251A+S253T had the same optimal temperature as RML 251 of 50°C while the other two variants, RML F251D+S253T and F251Q+S253T, had slightly lower optimal temperature of 45°C (Table 3).

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When the S253T mutation of RML 251 was removed, we noticed that the resulting RML F251N variant had comparable activity to RML 251 and had optimal temperature for olive oil hydrolysis at 50°C as well. Thus, for further analysis site saturation mutagenesis was performed at amino acid position 251 only.

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All variants generated by saturated mutagenesis at position 251, except RML F251W and RML F251Y, exhibited significant improvements in specific activity of between 109 to 281% compared to WT using olive oil as the substrate (Table 2). However, the F251W and F251Y were found to have slightly higher activity than the WT at 60°C and thus may still be useful in certain processes. By changing the substrate for hydrolysis to castor oil, glycerol trioctanoate, tributyrin, C12 methyl ester, C8/C10 methyl esters and vinyl laurate, specific activities of these F251 variants were between 109 to 922% higher than WT. RML F251Y, which demonstrated a small improvement of 22% at olive oil hydrolysis also had a more modest improvement of

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between 31-77% for the seven other substrates. RML F251W, which had similar activity as WT at hydrolysing olive oil, also had comparable activity at the hydrolysis of other substrates studied. (Table 2). Among these variants, eight maintained the optimal temperature of 55°C like in WT, while the remaining eleven variants had the same optimal temperature of 50°C as RML 251.

Table 2: Relative hydrolytic activity of RML variants with mutations at amino acid position 251. Olive oil, castor oil, glycerol trioctanoate, tributyrin, C12 methyl ester, C10 methyl ester, C8 methyl ester and vinyl laurate were used as substrates. The values are expressed as mean of triplicates.

	Relative activity (%)							
	Olive oil	Castor oil	Glycerol trioctanoate	Tributyrin	C12 methyl ester	C10 methyl ester	C8 methyl ester	Vinyl laurate
RML WT	100	100	100	100	100	100	100	100
RML 251 (F251N+S253T)	268	353	249	316	286	297	258	342
RML F251A+S253T	198							
RML F251D+S253T	202							
RML F251Q+S253T	228							
RML F251N	248	284	217	284	268	289	377	277
RML F251A	219	282	245	510	325	355	463	275
RML F251C	254	335	274	255	442	475	591	303
RML F251D	245	286	260	527	325	345	377	303
RML F251E	272	277	263	274	430	425	520	301
RML F251G	249	332	278	270	470	472	526	322
RML F251H	209	253	232	215	325	299	389	262
RML F251I	232	306	270	263	459	450	551	318
RML F251K	237	327	241	275	515	491	414	330
RML F251L	263	394	295	279	456	448	545	327
RML F251M	281	302	209	209	327	286	333	258
RML F251P	210	370	321	319	592	719	1022	329

RML F251Q	216	282	248	482	268	302	381	281
RML F251R	249	311	249	249	482	516	569	311
RML F251S	271	321	224	224	445	425	355	306
RML F251T	247	304	262	262	473	538	613	303
RML F251V	254	340	283	283	473	550	638	332
RML F251W	96	103	113	113	114	91	97	118
RML F251Y	122	162	141	141	168	139	181	157

The optimum temperature for hydrolysis of olive of the RML WT and variants are summarized in Table 3.

Table 3: Optimum temperature for olive oil hydrolysis for RML WT and variants.

	Optimum temperature (°C)
RML WT	55
RML 237	50
RML 243	40
RML 251	50
RML F251A+S253T	50
RML F251D+S253T	45
RML F251Q+S253T	45
RML F251N	50
RML F251A	50
RML F251C	50
RML F251D	50
RML F251E	50
RML F251G	50
RML F251H	55
RML F251I	55
RML F251K	55

RML F251L	55
RML F251M	50
RML F251P	55
RML F251Q	50
RML F251R	55
RML F251S	50
RML F251T	50
RML F251V	50
RML F251W	55
RML F251Y	55

All the mutation sites on RML237, RML243 and RML251 were introduced upstream of one of the residues of the catalytic triad, the His residue at position 257. For RML237, the mutations were located near a bend which is held in place by a disulphide bond (Figure 1a), and for RML243, the mutations were straddling the cysteine residue involved in the disulphide bond itself (Figure 1b). Hence, in these two variants, there may be potential disruptions to the disulphide bond formation, which may increase the flexibility of the strand involved in the formation of the substrate binding pocket. In RML251 (F251N+S253T), the two sites of mutations were located at a loop region in the vicinity of H257 and could thus potentially contribute to modifications in the substrate binding pocket as well (Figure 1c). Furthermore, the propeptide, which inhibits RML activity, could interact with the amino acid at position 251, therefore replacing the residue with a hydrophilic residue or a hydrophobic residue with less bulky side chain could have contributed to reduced interactions between the propeptide and mature protein and promoted higher activity. Together with the observed differences in substrate specificity and optimal temperature for activity, these variants seemed to have achieved higher hydrolytic activity through modifications of the structure leading to increased flexibility of substrate binding pocket and also through reduced interactions with the inhibiting propeptide. It is possible that the substitution of one or more of the amino acids at other positions that may come into contact with the propeptide may also reduce the interaction of the propeptide with the lipase. For example, modification of I204 to other amino acids that are

more hydrophobic, less hydrophobic, polar, negatively charged and positively charged, represented by Phe, Ala, Asn, Asp, Arg respectively, all showed improvements in hydrolytic activity of between 27-89% (Table 4). Modification of V254 to less hydrophobic, polar and negatively charged residues, represented by Ala, Asn and Asp, respectively, could also improve the hydrolytic activity by between 11-90% (Table 4).

Table 4: Relative hydrolytic activity of RML I204 and RML V254 variants using olive oil as substrate

	Relative activity (%)
RML WT	100
RML I204A	147
RML I204D	189
RML I204F	127
RML I204N	169
RML I204R	142
RML V254A	111
RML V254D	168
RML V254F	76
RML V254N	190
RML V254R	73

When an additional mutation of Asp to Gly was introduced at amino acid position 156 on the variants with mutations at 251, further improvements in activity were observed. RML F251W and RML F251Y mutants, which alone only showed slight improvement, became 149-717% more active than WT with this additional D156G mutation (Table 5). For all other variants with the D156G mutation, the most significant improvements were observed for the hydrolysis of C8-C12 methyl esters and castor oil (Table 5). The D156G mutation might have contributed to the higher activity due to modifications in the substrate binding pocket since Asp 156 is located on a helix structure where a Ser residue of the catalytic triad is also found.

Table 5: Relative hydrolytic activity of RML variants with D156G mutation on top of mutations at amino acid position 251. Olive oil, castor oil, glycerol trioctanoate, tributyrin,

C12 methyl ester, C10 methyl ester, C8 methyl ester and vinyl laurate were used as substrates. The values are expressed as mean of triplicates.

	Relative activity (%)							
	Olive oil	Castor oil	Glycerol trioctanoate	Tributylin	C12 methyl ester	C10 methyl ester	C8 methyl ester	Vinyl laurate
RML WT	100	100	100	100	100	100	100	100
RML 251+D156G	354	453	336	310	512	365	529	350
RML F251N+D156G	331	362	314	278	525	442	680	309
RML F251A+D156G	259	279	288	265	531	527	734	265
RML F251C+D156G	284	476	327	327	628	711	1057	333
RML F251D+D156G	247	433	301	290	536	575	750	287
RML F251E+D156G	224	357	283	266	509	510	798	256
RML F251G+D156G	234	321	296	270	534	537	773	280
RML F251H+D156G	220	419	323	324	609	639	891	320
RML F251I+D156G	213	359	296	284	562	580	854	289
RML F251K+D156G	219	384	311	251	587	566	794	302
RML F251L+D156G	243	362	310	294	568	530	789	311
RML F251M+D156G	257	338	298	266	492	437	699	293
RML F251Q+D156G	223	384	311	304	595	554	800	297
RML F251R+D156G	215	350	311	286	574	551	822	304
RML F251S+D156G	219	338	273	209	462	443	528	261
RML F251T+D156G	280	310	261	214	397	387	480	220
RML F251V+D156G	250	362	317	286	555	898	852	302
RML F251W+D156G	249	402	252	249	402	751	600	269
RML F251Y+D156G	281	433	254	255	473	817	683	300

RML mutagenesis and expression

- 5 To generate RML variants, site directed mutagenesis was used to introduce substitution into wild-type (WT) RML. The codon optimized sequence (SEQ ID No. 1) for WT RML was synthesized based on its amino acid sequence (UniProtKB - P19515) for optimal expression in *Pichia pastoris*. The propeptide region had been modified for improved expression in *Pichia*. Mutated RML were amplified from pAO815W-WT RML construct using AOX promoter and
- 10 terminator primers paired with primers designed to substitute the amino acid sequence at the

targeted sites (Table 6) and cloned into the pAO815W vector at the *Hind*III and *Eco*RI sites. The pAO815W vector was modified from pAO815 with the removal of the *Hind*III site within the 5'-AOX promoter region and its reintroduction downstream of the 5'-AOX promoter. The resulting pAO815W-RML variant constructs were transformed into *P. pastoris* GS115 by electroporation as described previously (Wu et al., 2004). For the expression of RML variants, cells from a single colony were cultured overnight at 30°C in shake flasks containing buffered glycerol-complex medium (BMGY; 1% yeast extract, 2% peptone, 100mM potassium phosphate, pH 6.0, 1.34% YNB, 4 x 10⁻⁵% biotin and 1% glycerol). On the following day, the cells were pelleted and resuspended in equal volumes of buffered methanol-complex medium (BMMY; 1% yeast extract, 2% peptone, 100mM potassium phosphate, pH 6.0, 1.34% YNB, 4 x 10⁻⁵% biotin and 0.5% methanol) with OD₆₀₀ normalized to that of the slowest growing culture, which is between 400-450. For the next three days, methanol (0.5% v/v) was supplemented to the cultures twice daily. On the 6th day, culture media containing the secreted RML were collected and clarified by centrifugation. Cultural liquid was analyzed using SDS-PAGE (Figure 2) to confirm for the presence of RML and its concentration was determined from Bradford assays using bovine serum albumin as a reference.

Lipase activity assay

Determination of specific activity for the different RML variants was performed using olive oil, castor oil, glycerol trioctanoate, tributyrin, C12 methyl ester, C8/C10 methyl esters and vinyl laurate emulsion as substrate. The emulsion was prepared by homogenizing the oil with 4% (w/v) poly vinyl alcohol 30 000 solution in a ratio of 1:3 using a knife homogenizer and used immediately. For the assay, 2 ml of the oil emulsion, 1.5 ml of dH₂O and 1 ml of 0.2 M Tris-HCl buffer, pH8.0 were first added to a 100 ml flat bottom flask and incubated in a 40°C water-bath with shaking at 150 rpm. For the determination of optimal pH for activity, the buffer was replaced with 0.2 M Bis-Tris at pH 6 and 7, and 0.2 M Glycine-Sodium hydroxide buffer at pH 9. After 5 min, 500 µl of the enzyme solution (10 µg/ml) was added to the reaction mixture and incubated for a further 15 mins. 5 ml of absolute ethanol was then added to terminate the reaction. The amount of free fatty acid released during the reaction was determined through titration with 50 mM NaOH using phenolphthalein as an indicator. The specific activity (U/mg) of each RML variant was calculated using the following equation:

$$\text{Specific activity (U/mg)} = \frac{(v - v_0) * C}{t * A}$$

v: Volume NaOH required for neutralization of reaction mix (ml)

v_0 : Volume of NaOH required for neutralization of blank (ml)

C: Concentration of NaOH ($\mu\text{mol/ml}$)

t : Reaction time (min)

A: Amount of RML added (mg)

5

1U corresponds to 1 μmol of free fatty acid released from the hydrolysis of olive oil in 1 min.

OPO and MLCT assay

To determine efficiency of RML251 for OPO and MLCT production, the lipase (RML251) was first immobilized on a hydrophobic resin. Non-limiting examples of a hydrophobic resin that may be used include: Lifetech™ ECR8806M, Lewatit® VP OC 1600 and AmberLite™ XAD™7HP Polymeric Adsorbent. Other resins such as ion exchange resin or mixed-mode resin may be used as well. A homogenized mixture containing palm oil and oleic acid was used for the OPO assay, and was melted and aliquoted into 2 ml tubes as the substrate and immobilized RML was added at 6% (w/v) dosage. The reaction was performed on a thermomixer at 60°C with shaking at 1500 rpm. At the required time point, the samples were centrifuged at 14 000 rpm for one min to separate the immobilized RML. The top fraction was stored at -20°C until analysis by LC-MS. The transesterification reaction between palm oil and oleic acid takes place in the absence of water or with minimal water present. A homogenized mixture containing high olein sunflower oil and medium chain triacylglycerol (with a mixture of C8 and C10 fatty acids, Wilfarester MCT) was used for the MLCT assay with the same procedure as for the OPO assay. GC-MS analysis was performed to determine the C32-C46 content.

In addition, it may be possible that the lipase variants described herein may be used to perform a transesterification reaction of two or more esters to produce the new ester product (transesterification product).

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Sequences

SEQ ID No. 1: *WT RML*

- 30 GAAGCCGAAGCTTCCATCGACGGAGGTATTAGAGCCGCTACTTCTCAGGAAATC
AACGAACTTACTTACTATACAACCTTTGTCAGCTAATTCTTACTGTAGAACTGTTAT
TCCTGGTGCTACTTGGGATTGCATACATTGTGACGCCACTGAAGATTTAAAGATA
ATTAAAACCTGGTCTACTTTGATTTACGACACTAACGCTATGGTTGCTAGAGGAG
ATTCCGAGAAGACTATTTATATCGTGTTTAGAGGTTCTTCATCTATTCGTAATTGG

ATCGCTGATTTGACATTCGTTCCAGTCTCTTACCCTCCAGTTTCTGGTACTAAGGT
TCACAAAGGATTTCTTGATTCTTATGGTGAAGTTCAAAACGAGTTGGTTGCTACT
GTCTTGATCAGTTTAAACAATACCCATCTTATAAGGTTGCTGTCACTGGTCACTC
TTGGGAGGTGCTACTGCCTTGCTGTGTGCTTTAGATTTATACCAGAGAGAGGAA
5 GGATTGTCTTCAAGTAACCTATTCTTGTACACTCAAGGTCAGCCTAGAGTTGGAG
ATCCAGCATTTGCTAATTATGTGGTTTCTACTGGTATTCCATATAGACGTACTGTT
AACGAAAGAGACATAGTACCACACTTGCCTCCAGCTGCCTTCGGATTTCTGCATG
CCGGTGAAGAGTACTGGATCACAGATAATTCTCCTGAAACCGTTCAAGTGTGTAC
ATCTGATTTAGAGACTTCCGACTGCTCTAACAGTATTGTTCCATTTACTTCAGTTC
10 TTGATCATTTGTCTTATTTTGAATTAACACCGGTTTGTGTACTTAA

SEQ ID No. 2: WT RML (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
15 GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCT

SEQ ID No. 3: RML237 (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
20 QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTNDTETSDCSNSIVPFTSVLDHLSYFGINTGLCT

SEQ ID No. 4: RML243 (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
25 QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETNSCTNSIVPFTSVLDHLSYFGINTGLCT

SEQ ID No. 5: RML251 (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
30 QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPNTTVLDHLSYFGINTGLCT

SEQ ID No. 6: RML 237+251 (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
5 GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTNDTETSDCSNSIVPNTTVLDHLSYFGINTGLCT

SED ID No. 7: RML 243+251 (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
10 QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETNSCTNSIVPNTTVLDHLSYFGINTGLCT

SEQ ID No. 8: RML F251A+S253T (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
15 DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPATTVLDHLSYFGINTGLCT

SEQ ID No. 9: RML F251D+S253T (mature protein)

20 SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPDTTVLDHLSYFGINTGLCT

25 SEQ ID No. 10: RML F251Q+S253T (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
30 QVCTSDLECSNASNSIVPQTTVLDHLSYFGINTGLCT

SEQ ID No. 11: RML F251N (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ

GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPNTSVLDHLSYFGINTGLCT

SEQ ID No. 12: RML F251A (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
5 DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLECSNASNSIVPATSVLDHLSYFGINTGLCT

SEQ ID No. 13: RML F251D (Mature protein)

10 SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPDTSVLDHLSYFGINTGLCT

15 SEQ ID No. 14: RML F251Q (Mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
20 QVCTSDLETSDCSNSIVPQTSVLDHLSYFGINTGLCT

SEQ ID No. 15: RML F251C (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
25 GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPCTSVLDHLSYFGINTGLCT

SEQ ID No. 16: RML F251E (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
30 QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPETSVDHLSYFGINTGLCT

SEQ ID No. 17: RML F251G (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
5 QVCTSDLETSDCSNSIVPGTSVLDHLSYFGINTGLCT

SEQ ID No. 18: RML F251H (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
10 GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPHTSVLDHLSYFGINTGLCT

SEQ ID No. 19: RML F251I (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
15 QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPITSVLDHLSYFGINTGLCT

SEQ ID No. 20: RML F251K (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
20 DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPKTSVLDHLSYFGINTGLCT

SEQ ID No. 21: RML F251L (mature protein)

25 SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPLTSVLDHLSYFGINTGLCT

30 SEQ ID No. 22: RML F251M (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ

GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPMTSVLDHLSYFGINTGLCT

SEQ ID No. 23: RML F251P (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
5 DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPPTSVDHLSYFGINTGLCT

SEQ ID No. 24: RML F251R (mature protein)

10 SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPRTSVLDHLSYFGINTGLCT

15 SEQ ID No. 25: RML F251S (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
20 QVCTSDLETSDCSNSIVPSTSVLDHLSYFGINTGLCT

SEQ ID No. 26: RML F251T (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
25 GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPTTSVLDHLSYFGINTGLCT

SEQ ID No. 27: RML F251V (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
30 QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPVTSVLDHLSYFGINTGLCT

SEQ ID No. 28: RML F251W (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
5 QVCTSDLETSDCSNSIVPWTSVLDHLSYFGINTGLCT

SEQ ID No. 29: RML F251Y (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
10 GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPYTSVLDHLSYFGINTGLCT

SEQ ID No. 30: RML I204A (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
15 QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDAVPHLPPAAFGLHAGEEYWITDNSPET
VQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCT

SEQ ID No. 31: RML I204D (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
20 DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDDVPHLPPAAFGLHAGEEYWITDNSPET
VQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCT

SEQ ID No. 32: RML I204F (mature protein)

25 SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDFVPHLPPAAFGLHAGEEYWITDNSPET
VQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCT

30 SEQ ID No. 33: RML I204N (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ

GQPRVGDPAFANYVVSTGIPYRRTVNERNVPHLPPAAFGFLHAGEEYWITDNSPET
VQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCT

SEQ ID No. 34: RML I204R (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
5 DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERNDRVPHLPPAAFGFLHAGEEYWITDNSPET
VQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCT

SEQ ID No. 35: RML V254A (mature protein)

10 SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERNDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPFTSALDHLSYFGINTGLCT

15 SEQ ID No. 36: RML V254D (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERNDIVPHLPPAAFGFLHAGEEYWITDNSPETV
20 QVCTSDLETSDCSNSIVPFTSDLDHLSYFGINTGLCT

SEQ ID No. 37: RML V254F (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
25 GQPRVGDPAFANYVVSTGIPYRRTVNERNDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPFTSFLDHLSYFGINTGLCT

SEQ ID No. 38: RML V254N (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
30 QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERNDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPFTSNLDHLSYFGINTGLCT

SEQ ID No. 39: RML V254R (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
5 QVCTSDLETSDCSNSIVPFTSRLDHLSYFGINTGLCT

SEQ ID No. 40: RML 251 + D156G (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ
10 GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPNTTVLDHLSYFGINTGLCT

SEQ ID No. 41: RML F251N + D156G (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
15 QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPNTSVLDHLSYFGINTGLCT

SEQ ID No. 42: RML F251A + D156G (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
20 DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLECSNASNSIVPATSVLDHLSYFGINTGLCT

SEQ ID No. 43: RML F251D + D156G (Mature protein)

25 SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPDTSVLDHLSYFGINTGLCT

30 SEQ ID No. 44: RML F251Q + D156G (Mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ

GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPQTSVLDHLSYFGINTGLCT

SEQ ID No. 45: RML F251C + D156G (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
5 DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALGLYQREEGLSSSNLFLYTQ
GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPCTSVLDHLSYFGINTGLCT

SEQ ID No. 46: RML F251E + D156G (mature protein)

10 SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALGLYQREEGLSSSNLFLYTQ
GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPETSVDHLSYFGINTGLCT

15 SEQ ID No. 47: RML F251G + D156G (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALGLYQREEGLSSSNLFLYTQ
GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
20 QVCTSDLETSDCSNSIVPGTSVLDHLSYFGINTGLCT

SEQ ID No. 48: RML F251H + D156G (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALGLYQREEGLSSSNLFLYTQ
25 GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPHTSVLDHLSYFGINTGLCT

SEQ ID No. 49: RML F251I + D156G (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
30 QNELVATVLDQFKQYPSYKVAVTGHS LGGATALLCALGLYQREEGLSSSNLFLYTQ
GQPRVGDPAPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPITSVDHLSYFGINTGLCT

SEQ ID No. 50: RML F251K + D156G (mature protein)

- SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
5 QVCTSDLETSDCSNSIVPKTSVLDHLSYFGINTGLCT
SEQ ID No. 51: RML F251L + D156G (mature protein)
SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ
10 GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPLTSVLDHLSYFGINTGLCT
SEQ ID No. 52: RML F251M + D156G (mature protein)
SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
15 QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPMTSVLDHLSYFGINTGLCT
SEQ ID No. 53: RML F251P + D156G (mature protein)
SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
20 DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPPTSVDHLSYFGINTGLCT
SEQ ID No. 54: RML F251R + D156G (mature protein)
25 SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPRTSVLDHLSYFGINTGLCT
30 SEQ ID No. 55: RML F251S + D156G (mature protein)
SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ

GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPSTSVDHLSYFGINTGLCT

SEQ ID No. 56: RML F251T + D156G (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
5 DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPTTSVDHLSYFGINTGLCT

SEQ ID No. 57: RML F251V + D156G (mature protein)

10 SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPVTSTVDHLSYFGINTGLCT

15 SEQ ID No. 58: RML F251W + D156G (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ
GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAGFLHAGEEYWITDNSPETV
20 QVCTSDLETSDCSNSIVPWTSTVDHLSYFGINTGLCT

SEQ ID No. 59: RML F251Y + D156G (mature protein)

SIDGGIRAATSQEINELTYTTLSANSYCRTVIPGATWDCIHCDATEDLKIIKTWSTLIY
DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEV
QNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALGLYQREEGLSSSNLFLYTQ
25 GQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAGFLHAGEEYWITDNSPETV
QVCTSDLETSDCSNSIVPYTSTVDHLSYFGINTGLCT

Table 6: List of primers used

	Forward primer	Reverse Primer
AOX	GACTGGTTCCAATTGACAA CG (SEQ ID No. 60)	GCAAATGGCATTCTGACATCC (SEQ ID No. 61)

RML237	GTGTACAAACGATACTGAG ACTTCCGACTGCTCTAA (SEQ ID No. 62)	AAGTCTCAGTATCGTTTGTAC ACACTTGAACGGTTTC (SEQ ID No. 63)
RML243	TTAGAGACTTCCAAGTCA CTAACAGTATTGTTCCATTT (SEQ ID No. 64)	AGTGCAGTTGGAAGTCTCTAA ATCAGATGTAC (SEQ ID No. 65)
RML251	GTATTGTTCCAAACACTAC TGTTCTTGATCATTTGTCT (SEQ ID No. 66)	GAACAGTAGTGTTTGGAAACA ATACTGTTAGAGCAG (SEQ ID No. 67)
RML F251N	GTATTGTTCCAAACACTTC AGTTCTTGATCATTTGTCT (SEQ ID No. 68)	GAAGTGAAGTGTTTGGAAACA ATACTGTTAGAGCAGT (SEQ ID No. 69)
RML F251A	GTATTGTTCCAGCTACTTCA GTTCTTGATCATTTGTCT (SEQ ID No. 70)	GAAGTGAAGTAGCTGGAACA ATACTGTTAGAGCAG (SEQ ID No. 71)
RML F251D	GTATTGTTCCAGATACTTCA GTTCTTGATCATTTGTCT (SEQ ID No. 72)	GAAGTGAAGTATCTGGAACA ATACTGTTAGAGCAG (SEQ ID No. 73)
RML F251Q	GTATTGTTCCACAAACTTC AGTTCTTGATCATTTGTCT (SEQ ID No. 74)	GAAGTGAAGTTTGTGGAACA ATACTGTTAGAGCAG (SEQ ID No. 75)
RML F251C	CGACTGCTCTAACAGTATT GTTCCATGTACTTCAGTTCT TGATCATTTGTCT (SEQ ID No. 76)	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)
RML F251E	CGACTGCTCTAACAGTATT GTTCCAGAACTTCAGTTC TTGATCATTTGTCT (SEQ ID No. 78)	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)

RML F251G	CGACTGCTCTAACAGTATT GTTCCAGGTACTTCAGTTCT TGATCATTTGTCT (SEQ ID No. 79)	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)
RML F251H	CGACTGCTCTAACAGTATT GTTCCACATACTTCAGTTCT TGATCATTTGTCT (SEQ ID No. 80)	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)
RML F251I	CGACTGCTCTAACAGTATT GTTCCAATTACTTCAGTTCT TGATCATTTGTCT (SEQ ID No. 81)	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)
RML F251K	CGACTGCTCTAACAGTATT GTTCCAAAGACTTCAGTTC TTGATCATTTGTCT (SEQ ID No. 82)	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)
RML F251L	CGACTGCTCTAACAGTATT GTTCCATTGACTTCAGTTCT TGATCATTTGTCT (SEQ ID No. 83)	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)
RML F251M	CGACTGCTCTAACAGTATT GTTCCAATGACTTCAGTTCT TGATCATTTGTCT (SEQ ID No. 84)	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)
RML F251P	CGACTGCTCTAACAGTATT GTTCCACCAACTTCAGTTCT TGATCATTTGTCT (SEQ ID No. 85)	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)
RML F251R	CGACTGCTCTAACAGTATT GTTCCAAGAACTTCAGTTC	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)

	TTGATCATTTGTCT (SEQ ID No. 83)	
RML F251S	CGACTGCTCTAACAGTATT GTTCCATCTACTTCAGTTCT TGATCATTTGTCT (SEQ ID No. 87)	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)
RML F251T	CGACTGCTCTAACAGTATT GTTCCAATACTTCAGTTCT TGATCATTTGTCT (SEQ ID No. 88)	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)
RML F251V	CGACTGCTCTAACAGTATT GTTCCAGTTACTTCAGTTCT TGATCATTTGTCT (SEQ ID No. 89)	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)
RML F251W	CGACTGCTCTAACAGTATT GTTCCATGGACTTCAGTTCT TGATCATTTGTCT (SEQ ID No. 90)	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)
RML F251Y	CGACTGCTCTAACAGTATT GTTCCATACACTTCAGTTCT TGATCATTTGTCT (SEQ ID No. 91)	TGGAACAATACTGTTAGAGCA GTCG (SEQ ID No. 77)
RML I204A	CGTACTGTTAACGAAAGAG ACGCTGTACCACACTTGCC TCCAGC (SEQ ID No. 92)	GTCTCTTTCGTTAACAGTACG TCTATATG (SEQ ID No. 93)
RML I204D	CGTACTGTTAACGAAAGAG ACGATGTACCACACTTGCC TCCAGC (SEQ ID No. 94)	GTCTCTTTCGTTAACAGTACG TCTATATG (SEQ ID No. 93)

RML I204F	CGTACTGTTAACGAAAGAG ACTTTGTACCACACTTGCCT CCAGC (SEQ ID No. 95)	GTCTCTTTCGTTAACAGTACG TCTATATG (SEQ ID No. 93)
RML I204N	CGTACTGTTAACGAAAGAG ACAACGTACCACACTTGCC TCCAGC (SEQ ID No. 96)	GTCTCTTTCGTTAACAGTACG TCTATATG (SEQ ID No. 93)
RML I204R	CGTACTGTTAACGAAAGAG ACAGAGTACCACACTTGCC TCCAGC (SEQ ID No. 97)	GTCTCTTTCGTTAACAGTACG TCTATATG (SEQ ID No. 93)
RML V254A	CTAACAGTATTGTTCCATTT ACTTCAGCTCTTGATCATTT GTCTTATTTTGGAATTAAC (SEQ ID No. 98)	TGAAGTAAATGGAACAATAC TGTTAGAGC (SEQ ID No. 99)
RML V254D	CTAACAGTATTGTTCCATTT ACTTCAGATCTTGATCATTT GTCTTATTTTGGAATTAAC (SEQ ID No. 100)	TGAAGTAAATGGAACAATAC TGTTAGAGC (SEQ ID No. 99)
RML V254F	CTAACAGTATTGTTCCATTT ACTTCATTTCTTGATCATTT GTCTTATTTTGGAATTAAC (SEQ ID No. 101)	TGAAGTAAATGGAACAATAC TGTTAGAGC (SEQ ID No. 99)
RML V254N	CTAACAGTATTGTTCCATTT ACTTCAAACCTTGATCATTT GTCTTATTTTGGAATTAAC (SEQ ID No. 102)	TGAAGTAAATGGAACAATAC TGTTAGAGC (SEQ ID No. 99)
RML V254R	CTAACAGTATTGTTCCATTT ACTTCAAGACTTGATCATTT GTCTTATTTTGGAATTAAC (SEQ ID No. 103)	TGAAGTAAATGGAACAATAC TGTTAGAGC (SEQ ID No. 99)

The same reverse primers (SEQ ID Nos. 77, 93 and 99) may be used for the substitution of phenylalanine at position 251, isoleucine at position 204 and valine at position 254 as shown above.

Claims

1. A lipase variant comprising a first substituent of a lipase sequence of SEQ ID No. 2, wherein a position of the first substituent in SEQ ID No. 2 is selected from the group consisting of position 251, position 204, position 254, position 237, and position 243.
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2. The lipase variant according to claim 1 wherein the first substituent is less hydrophobic than phenylalanine.
3. The lipase variant according to any one of claims 1 to 2 wherein the position of the first
10 substituent is at position 251 and the first substituent is selected from the group consisting of asparagine, alanine, cysteine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, lysine, leucine, methionine, proline, glutamine, arginine, serine, threonine, valine, tryptophan and tyrosine.
- 15 4. The lipase variant according to claim 1 wherein the position of the first substituent is at position 204 and the first substituent is selected from the group consisting of more hydrophobic, less hydrophobic, polar and uncharged, negatively charged and positively charged, wherein the charge is determined with respect to the first substituent at a pH of 7.
- 20 5. The lipase variant according to claim 4 wherein the position of the first substituent is at position 204 and the first substituent is selected from the group consisting of phenylalanine, leucine, methionine, tyrosine, tryptophan, alanine, valine, glycine, methionine, proline, serine, threonine, asparagine, glutamine, aspartic acid, glutamic
25 acid, arginine, histidine, and lysine.
6. The lipase variant according to claim 5 wherein the first substituent is selected from the group consisting of alanine, aspartic acid, phenylalanine, asparagine, and arginine.
- 30 7. The lipase variant according to claim 1 wherein the position of the first substituent is at position 254 and the first substituent is selected from the group consisting of less hydrophobic, polar, and negatively charged, wherein the charge is determined with respect to the first substituent at a pH of 7.

8. The lipase variant according to claim 7 wherein the position of the first substituent is at position 254 and the first substituent is selected from the group consisting of methionine, tyrosine, cysteine, alanine, glycine, serine, threonine, asparagine, glutamine, aspartic acid, and glutamic acid.
- 5 9. The lipase variant according to claim 8 wherein the first substituent is selected from the group consisting of alanine, aspartic acid, and asparagine.
- 10 10. The lipase variant according to any one of claims 1 to 9 comprising a second substituent of the lipase sequence of SEQ ID No.2, wherein the position of the second substituent in SEQ ID No. 2 is position 253, wherein the first substituent is preferably at position 251.
- 15 11. The lipase variant according to claim 10 wherein the first substituent is at position 251 and selected from the group consisting of asparagine, alanine, aspartic acid, and glutamine.
- 20 12. The lipase variant according to any one of claims 1 to 11 comprising a third substituent of the lipase sequence of SEQ ID No.2, wherein the position of the third substituent in SEQ ID No. 2 is position 156 wherein the first substituent is preferably at position 251.
- 25 13. The lipase variant according to any one of claims 1 to 12 wherein the first substituent is at position 251, the lipase variant comprising a fourth substituent and a fifth substituent, wherein the fourth substituent and the fifth substituent is either at positions 237 and 239 respectively or positions 243 and 245 respectively.
- 30 14. The lipase variant according to claim 1 comprises a sequence selected from the group consisting of SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28, SEQ ID No. 29, SEQ ID No. 30, SEQ ID No. 31, SEQ ID No. 32, SEQ ID No. 33, SEQ ID No. 34, SEQ ID No. 35, SEQ ID No. 36, SEQ ID No. 37, SEQ ID No. 38, SEQ ID

No. 39, SEQ ID No. 40, SEQ ID No. 41, SEQ ID No. 42, SEQ ID No. 43, SEQ ID No. 44, SEQ ID No. 45, SEQ ID No. 46, SEQ ID No. 47, SEQ ID No. 48, SEQ ID No. 49, SEQ ID No. 50, SEQ ID No. 51, SEQ ID No. 52, SEQ ID No. 53, SEQ ID No. 54, SEQ ID No. 55, SEQ ID No. 56, SEQ ID No. 57, SEQ ID No. 58, and SEQ ID No. 59.

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15. The lipase variant according to claim 1 comprising a sixth substituent of a lipase sequence of SEQ ID No. 2, wherein the first substituent and the sixth substituent are at or proximal to a surface loop region of the lipase sequence, wherein the first substituent is asparagine and the sixth substituent is threonine, the sixth substituent being two amino acid positions away from the first substituent.
16. The lipase variant according to claim 15 wherein the first substituent and sixth substituent is selected from the group consisting of S237N and L239T, D243N and S245T, and F251N and S253T.
17. A method comprising providing the lipase variant according to any one of claims 1 to 16, a first ester and a reactant, wherein the reactant is selected from the group consisting of water, an acid and a second ester; and forming a reaction product between the first ester and the reactant with the aid of the lipase variant under suitable reaction conditions.
18. The method according to claim 17 wherein the first ester is a fatty acid ester, preferably a triacylglyceride.
19. The method according to claim 18 wherein the fatty acid ester or triacylglyceride is selected from the group consisting of olive oil, castor oil, sunflower oil, high oleic sunflower oil, rapeseed oil, high oleic rapeseed oil, palm oil, palm fraction enriched in tripalmitin, shea butter, shea olein, canola oil, glycerol trioctanoate, tributyrin, methyl octanoate, methyl decanoate, methyl dodecanoate, and vinyl laurate.
20. The method according to any one of claims 17 to 19 wherein the reactant is a medium chain fatty acid or ester.
21. The method according to claim 20 wherein the reactant is selected from the group consisting of octanoate ester, decanoate ester, dodecanoate ester, and mixtures thereof.

22. The method according to any one of claims 20 to 21 wherein the reaction product is a triacylglyceride with medium chain fatty acids at a 1,3-position of the triacylglyceride and a long chain fatty acid at a 2-position of the triacylglyceride.
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23. The method according to any one of claims 17 to 22 wherein the reactant is a long chain fatty acid or ester.
24. The method according to claim 17 wherein the first ester is palm oil and the reactant is oleic acid, oleic ester, linoleic acid or linoleic ester.
- 10
25. The method according to claim 24 wherein the first ester is palm fraction enriched in tripalmitin.
- 15
26. The method according to claim 19 wherein the fatty acid ester or triacylglyceride is selected from the group consisting of olive oil, high oleic sunflower oil, and high oleic rapeseed oil, and the reactant is stearic acid or stearic ester.
27. The method according to claim 26 wherein the reaction product is 1,3-distearoyl-2-oleoyl glycerol.
- 20
28. The method according to any one of claims 17 to 27 wherein the lipase variant is SEQ ID No. 5.
- 25
29. The method according to any one of claims 17 to 28 wherein water is provided as an aqueous buffer.
30. The method according to any of claims 17 to 29 wherein the lipase variant is immobilized on a resin.
- 30
31. The method according to any of claims 17 to 30 wherein the first ester and the reactant are in a liquid phase or a solution phase under the suitable reaction conditions.

32. The method according to any of claims 17 to 31 wherein the reactant is the second ester, and the reaction product is a transesterification product of the first ester and the second ester.
- 5 33. A product obtained from the method according to any of claims 17 to 32.