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(54) Title: METHODS AND COMPOSITIONS TO REDUCE SERUM LEVELS OF TRIACYLGLYCERIDES IN HUMAN BEINGS USING A FUNGAL LIPASE

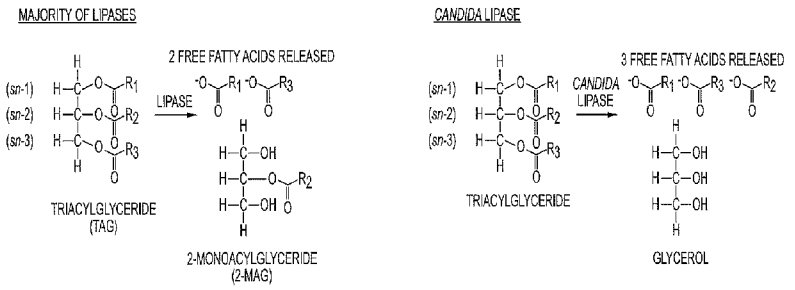

FIG. 1

(57) Abstract: The invention relates to methods and compositions for reducing serum levels of triacylglycerides in human subjects. In particular, the invention relates to the oral administration of an effective amount of a fungal lipase formulation, to a human subject having borderline-high or high serum levels of triacylglycerides, for a time period sufficient to reduce serum triacylglyceride levels.

METHODS AND COMPOSITIONS TO REDUCE SERUM LEVELS OF TRIACYLGLYCERIDES IN HUMAN BEINGS USING A FUNGAL LIPASE

Background of the Invention

1. Field of the Invention

[001] The invention relates to methods and compositions for reducing serum levels of triacylglycerides in human subjects. In particular, the invention relates to the oral administration of an effective amount of a fungal lipase formulation, to a human subject having high or borderline-high serum levels of triacylglycerides, for a time period sufficient to reduce serum triacylglyceride levels.

2. Description of Related Art

Triacylglyceride Levels

[002] A recent study by Ford *et al.*(Arch Intern Med, 169(6):572-578, 2009), at the Centers for Disease Control (CDC), involving 5,610 participants, showed that 33.1 percent of the participants had serum triacylglyceride levels defined as borderline high (>150 milligrams per deciliter). Nearly 18 percent had serum triacylglyceride levels defined as high (>200 milligrams per deciliter). High triacylglyceride levels may lead to atherosclerosis, which can increase the risk of heart attack and stroke. Persons with high triacylglyceride levels often have additional conditions that can increase their chances of developing heart disease, such as obesity and diabetes.

[003] Hypertriglyceridemia is a human condition characterized by the presence of high serum levels of triacylglycerides. It has been associated with obesity, but can also be induced by other factors, independent of overall body fat (CT Johansen and RA Hegele, Curr. Opin. Lipidol., 22(4):247-253, 2011). The presence of single nucleotide polymorphisms in cellular genes such APOA5, APOB, GCKR, and LPL can induce severe hypertriglyceridemia in human beings. It is thought that more than 20% of the susceptibility to hypertriglyceridemia is caused by genetic

mutations. Endothelial cell dysfunction and adipose cell dysfunction can lead to hypertriglyceridemia (HW Breuer Curr. Med. Res. Opin. 17(1):60-73, 2001; AP van de Woestijne et. al. Obes. Rev. 10.1111 (epub ahead of print) July 12, 2011. Hypertriglyceridemia can be induced by specific drugs. For example, interferon alfa-2b and asparaginase have been shown to induce hypertriglyceridemia by two unique biochemical mechanisms (YK Keung et. al. South. Med. J. 92(9):912-914, 1999). Taken together, these studies indicate that high serum levels of triacylglycerides can be induced in human beings by multiple factors independent of obesity.

[004] High serum levels of triacylglycerides (commonly called triglycerides; also referred to herein as "TAG") have been identified as one of several lipid abnormalities commonly associated with patients that have type 2 diabetes (Bitzur *et al.*, Diabetes Care 32 (suppl 2): S373-S377, 2009). It is currently unknown what mechanism(s) causes elevated serum TAG levels in these patients. The effect could be multi-factorial in nature, with contributions by diminished suppressive effects of insulin action, impaired clearance of lipid particles containing TAG, and the conscious dietary decisions by patients with type 2 diabetes to reduce carbohydrate caloric intake, replacing it with more meat and fats in their diets. Several prospective studies have identified hypertriglyceridemia as an independent predictor of type 2 diabetes. Recently, profiling of TAG molecules has been employed to help predict those that will acquire type 2 diabetes in the future (Rhee *et al.* J. Clin. Invest. 121(4):1402-1411, 2011). In view of these studies, clinicians monitor the level of triglycerides in a patient's serum, and researchers are looking for methods of lowering serum triglyceride levels.

Lipases

[005] Lipases are enzymes that the body uses to break down fats to aid in the digestion of food. The specificity of lipases is derived from the molecular properties of the lipase, the structure of a potential substrate, and factors that affect the binding of the lipase to a substrate (Jensen RG *et al.* Lipids, 18(3):239-252, 1983). One of the most important lipases in the human body is pancreatic

lipase. Pancreatic lipase breaks down triacylglycerides from ingested fats into smaller components that can be readily absorbed by intestinal cells (i.e., enterocytes), such as free fatty acids (FA) and 2-monoacylglyceride (2-MAG) (Pham, C.T. and Tso, P., Frontiers in Bioscience 6:d299-319, 2001; Levy *et al.* FASEB J. 9:626-635, 1995). The enterocyte synthesizes the FA and 2-MAG components into triacylglycerides (TAG) primarily via the Monoacylglyceride Pathway. The newly synthesized TAG are packaged into chylomicrons and then exocytosed into intracellular spaces and nearly lymphatic vessels. Chylomicrons containing TAG are ultimately distributed throughout the body for energy production and synthetic processes.

[006] Historically, attempts to enhance/supplement lipid digestion have focused on oral administration of supplemental amounts of pancreatic lipase. However, naturally supplied pancreatic lipase is secreated directly into the intestine via the bile duct, while oral supplements must first transit the stomach. Pancreatic lipase is not stable in the acidic environment of the stomach whether it is empty (pH 1-2) or full (pH 3-4), and rapidly loses its enzymatic activity during exposure to these low pH conditions. Previous attempts to overcome the instability of pancreatic lipase activity at acid pH have shown unsatisfactory results. Approaches have included using enteric-coated tablets of pancreatic lipase (Graham DY, New England Journal of Medicine 296:1314-1317, 1977), microsphere-encapsulated pancreatic lipase (see U.S. Patents 5,260,074; 5,324,514; 5,352,460; 5,405,621), and cross-linked lipase crystals (see U.S. Patent Applications 2001/0046493 and 2003/0017144).

[007] Accordingly, there remains a need for a lipase formulation that can be easily ingested and exert effective lipase activity, first in the acid pH environment of the full stomach and then in the neutral pH environment of the intestine.

Summary of the Invention

[008] The present invention provides lipase formulations that are stable and active in acidic and neutral pH environments, and that also break down dietary fats such as triacylglycerides into fatty acids (FA) and glycerol. By fully digesting triacylglycerides to FA and glycerol, which is beyond the scope of pancreatic lipase digestion, the lipase formulations of the invention enhance lipid digestion. The inventors have determined that these lipase formulations can significantly lower serum triacylglyceride levels in human beings.

[009] As such, the invention provides improved lipase compositions useful in treating human beings with high and borderline-high serum levels of triacylglycerides, as well as methods of reducing serum triacylglyceride levels in humans by administering these compositions.

[010] In particular, the invention provides for methods of reducing serum triacylglyceride levels in a human subject by administering a composition comprising an effective amount of a fungal lipase that (i) exhibits at least 50% of its maximum activity over the pH range of 2.0 to 8.0; and (ii) cleaves all three ester sites of a triglyceride, to a human subject in need thereof.

[011] In one embodiment, the method involves administering the composition periodically and over a time period sufficient to reduce serum triacylglycerides by at least 20% compared to the serum triacylglycerides of the human subject before administering the composition to the human. For example, the composition may be administered at each meal, or a majority of meals, for at least a week or a month.

[012] In another embodiment, the method reduces serum triacylglycerides in a human subject having at least 150 mg/dL of serum triacylglycerides. In another embodiment, the human subject is at risk for or has diabetes.

[013] In particular embodiments, the effective amount of the lipase may be, for example, at least 15,000 FIP units/meal, at least 180,000 FIP units per gram of protein, or at least 2000 FIP units per

gram of fat consumed by said human subject. Suitable fungal lipase may be a lipases derived from the *Candida* family such as a *Candida rugosa* (also known as *Candida cylindracea*) or *Candida antarctica* lipase.

Brief Description of the Drawings

[014] Figure 1 is a two-part diagram comparing the enzymatic actions of lipases having 1,3-specificity on triacylglycerides, and the actions of non-specific lipases on triacylglycerides.

[015] Figure 2 is a drawing showing the normal absorption of lipids by enterocytes and synthesis of triacylglycerides. Figure Abbreviations: Pancreatic Lipase (PL); Free Fatty Acid (FA); 2-Monoacylglyceride (2-MAG); Diacylglyceride (DAG); Triacylglyceride (TAG); Coenzyme A (CoA); Glycerol 3-Phosphate (Glycerol-3P).

[016] Figure 3 is a drawing showing the effects of *Candida rugosa* lipase on absorption of lipids by enterocytes and synthesis of triacylglycerides. Figure Abbreviations: Pancreatic Lipase (PL); *Candida* Lipase (CL); Free Fatty Acid (FA); Significantly Reduced Levels of 2- Monoacylglyceride [2-MAG]; Diacylglyceride (DAG); Triacylglyceride (TAG); Coenzyme A (CoA); Glycerol 3-Phosphate (Glycerol-3P).

[017] Figure 4 contains two graphs showing the effect of pH on the activity (Figure 4A) and stability (Figure 4B) of the lipase preparation derived from *Candida rugosa.*

[018] Figure 5 contains four graphs showing the effect of pH on the enzymatic activity (Figure 5A) and stability (Figure 5B) of the lipase preparation derived from *Aspergillus niger*, and the effect of pH on the enzymatic activity (Figure 5C) and stability (Figure 5D) of the lipase preparation derived from *Rhizopus oryzae.* The lipases were analyzed as described for Figures 4A and 4B.

[019] Figure 6 is a graph comparing the temporal digestive effects of lipases from 4 sources (pig pancreas; *Aspergillus niger*; *Rhizopus oryzae;* and *Candida rugosa*) using soybean oil at pH 4. The effectiveness of action was measured by decreased HPLC detection of triacylglycerides following

digestion into constitutive components (i.e., FA, 2-MAG, glycerol). In control preparations without lipase, an average of 874±8 mg/ml of triacylglycerides was detected at the four reaction time points studied.

[020] Figure 7 is a graph comparing the temporal production of glycerol by lipases of four sources (pig pancreas; *Aspergillus*; *Rhizopus;* and *Candida rugosa*) using soybean oil at pH 4. The effectiveness of action was measured by increased HPLC detection of glycerol following digestion of soybean oil. In control preparations without lipase, no glycerol was detected at the four reaction time points studied.

[021] Figure 8 is a concentration-response curve showing the specificity of the reaction by comparing increasing activity of the *Candida rugosa* lipase preparation to the production of glycerol. The *Candida rugosa* lipase needs to be used at an activity concentration capable of totally digesting TAG to FA and glycerol, and thus, achieve the invention's reductive effects *in vivo* on serum levels of TAG.

[022] Figure 9 is a graph comparing the action of 4 sources of lipases on the breakdown of triacylglycerides and the subsequent production of glycerol from olive oil at pH 4 demonstrating the generality of the observed actions of *Candia* lipase on other triacylglyceride sources.

Detailed Description of Preferred Embodiments of the Invention

[023] The invention provides improved enzyme compositions and methods for treatment of human beings with higher than normal serum levels of triacylglycerides. In particular, the invention provides for the administration of lipases that are (1) active and stable throughout the digestion (i.e., active and stable in acidic, acidic-to-neutral, and neutral pH); and (2) will hydrolyze all three fatty acid bonds of a triacylglyceride, in amounts effective to reduce serum levels of triacylglycerides. The invention provides effective amounts of the lipase compositions, and it directs these compositions at human populations that will benefit from their administration.

Lipase Activity and Stability

[024] The invention provides for the use of lipases that are active and stable in the acid environment of stomach and can begin digestion of dietary triacylglycerides there. The lipases are also active and stable in acidic-to-neutral pH and neutral pH conditions. As such, the lipases are active and stable throughout digestion. Examples of such lipases include lipases from the *Candida* family (e.g., *C. rugosa* and *C. antarctica*). In one embodiment, the lipase exhibits at least 50% of its maximum activity over the pH range of 2.0 to 8.0. It will be understood that other activity and stability ranges may be derived from, and are supported in, the Figures herein or from U.S. Publication No. 2008/0279839, which is hereby incorporated by reference.

Cleavage Activity of Lipase

[025] The lipases used in the compositions and methods of the invention are capable of cleaving all three ester sites (sn-1, sn-2, and sn-3) of triacylglycerides. The lipases can cleave fatty acid components off of the TAG at these three sites whether they are short, medium, or long in length, and/or whether they are unsaturated or saturated.

[026] It is understood that most lipases only cleave the sn-1 and/or sn-3 external sites of triacylglycerides and are unable to cleave all three ester sites. This results in the generation of up to two free fatty acid molecules and 2-monoacylglyceride (2-MAG) from triacylglyceride catabolism by these lipases, i.e., these lipases incompletely digest triacylglycerides. *See* Figure 1, first panel. In contrast, preferred lipases of this invention, such as those derived from *Candida rugosa* and *Candida antarctica*, are able to fully digest triacylglycerides to three free fatty acid molecules and a glycerol backbone. *See* Figure 1, second panel.

[027] One study characterized the substrate specificity of 25 lipases from numerous sources and found only 6 lipases (mainly *Candida* and *Geotrichum* lipases) that showed reactivity towards the sn-2 region of triacylglycerides (Rogalska *et al.,* Chirality, 5:24-30, 1993). Nearly all of the sn-2 position-

reactive lipases analyzed preferred to hydrolyze the external sn-1 and sn-3 positions, rather the internal sn-2 position of triacylglycerides, except for the *Candida antarctica* lipase, which showed a slight preference for the sn-2 position. Some of the sn-2 position-reactive lipases were derived from *Geotrichum* species.

[028] Recent work has indicated that some *Geotrichum*-derived lipases are stable within a pH range of 6.5-8.0 (e.g., Gopinath S.C.B.,*et al.* World J Review of MICB and Biotechnol., 19(7):681-689, 2003) suggesting that these particular lipases may be poorly effective in the acidic pH of the stomach for triacylglyceride digestion. *Geotrichum*-derived lipases that are stable at acidic pH, however, may be helpful and show some benefit at achieving improved digestion of triacylglycerides. Other microbial lipases, such as the one derived from *Aspergillus niger*, are stable at acidic pH, but will only attack the sn-1 and sn-3 positions of triacylglycerides, and also exhibit much greater substrate restriction than the more substrate-promiscuous *Candida rugosa* lipase, the preferred lipase of the invention. Accordingly, the invention contemplates the use of lipases, other than those derived from the *Candida* family, that cleave all three ester sites (sn-1, sn-2, and sn-3) of triacylglyceride.

Non-Binding Mechanistic Theory

[029] The human body, through pancreatic lipase, breaks down triacylglycerides from ingested fats into free fatty acids (FA) and 2-monoacylglyceride (2-MAG). Intestinal enterocytes synthesize the FA and 2-MAG components into triacylglycerides primarily via the Monoacylglyceride Pathway. TAG are ultimately distributed throughout the body for energy production and synthetic processes.

[030] Figure 2 depicts the normal digestion of lipid mixed micelles by pancreatic lipase. During the normal digestive process, mixed micelles are formed by the combination of bile salts with triacylglycerides to increase solubility of the lipid components. In the intestinal lumen, pancreatic lipase (PL) is assisted in anchoring to these mixed micelles by the action of colipase, an accessory protein secreted by the pancreas. Pancreatic lipase digests the triacylglycerides contained in mixed

micelles to fatty acids (FA) and 2-monoacylglyceride (2-MAG). These two digestion products (FA and 2-MAG) are quickly absorbed by the enterocyte and primarily shuttled to the Monoacylglyceride Pathway for reformation into TAG.

[031] Some studies have shown that 2-MAG can isomerize to the 1-monacylglyceride conformation *in vitro*. This phenomenon is not thought to be physiologically significant because absorption of 2-MAG by the enterocyte is too rapid for the isomerization process to occur. Normally, 70-75% of triacylglycerides produced by the enterocyte are synthesized via the Monoacylglyceride Pathway in the smooth endoplasmic reticulum (Mansbach and Parthasarathy, J. Lipid Research, 23:1009-1019, 1982; Levy *et al.* FASEB J, 9:626-635, 1995). After subsequent addition of apoproteins and other components, TAG-rich pre-chylomicrons are transported towards the basolateral region of the enterocyte and exocytosed into the extracellular space where the mature chylomicrons make their way to the lymphatics and are distributed throughout the body.

[032] A second TAG-synthetic pathway is also active in enterocytes. The Phosphatidic Acid Pathway synthesizes far smaller amounts of TAG compared to the Monoacylglyceride Pathway, mainly because it is constrained by the scarceness of its key pathway substrate, glycerol 3-phosphate (glycerol-3P). Glycerol-3 phosphate is usually imported by the enterocyte from other sources, such as from hepatic production. It has been shown that both the Monoacylglyceride Pathway and Phosphatidic Acid Pathway converge at the smooth endoplasmic reticulum and produce essentially identical TAG-rich chylomicron particles for export (Yang, L-Y. and Kuksis, A., J. Lipid Research 32:1173-1186, 1991).

[033] Without wishing to be bound, the inventors theorize that the lipase compositions described herein reduce the serum levels of triacylglycerides, by the concerted actions of the lipase (e.g., *Candida* lipase) and native pancreatic lipase on dietary lipids *in vivo*. By working together, the lipases are better able to completely digest most dietary triacylglycerides to three molecules of fatty acids

and one molecule of glycerol. A suggested scenario of the biochemical events involved is shown in Figure 3.

[034] During the enhanced digestive process, pancreatic lipase (PL) and *Candida* lipase (CL) digest triacylglycerides contained in mixed micelles to fatty acids (FA) and 2-monoacylglyceride (2-MAG). *Candida* lipase can then further digest the 2-MAG product into an additional FA molecule and free glycerol molecule. These two digestion products (FA and glycerol) are quickly absorbed by the intestinal enterocyte. The complete digestion of TAG causes a substantial decrease in the activity of the Monoacylglyceride Pathway since its substrate; 2-MAG has been greatly depleted. It has been shown in a previous study that the Phosphatidic Acid Pathway can become the major synthetic route of TAG production in the absence of 2-MAG (Mansbach and Parthasarathy, 1982). This synthetic compensation depends on the availability of the pathway substrate, glycerol-3 phosphate. Enterocyte glycerol kinase activity can produce the Phosphatidic Acid Pathway's substrate, glycerol-3 phosphate (glycerol-3P). The activity of glycerol kinase in the enterocyte, however, has been shown to be limited and most glycerol is believed to flow through and out the enterocyte to be used by other cellular synthetic pathways in the body. Most glycerol-3 phosphate is thought to be imported into the enterocyte from other sources. Thus, the concerted actions of native pancreatic lipase and *Candida* lipase on dietary lipids may lead to reduced amounts of triacylglycerides being synthesized and packaged into chylomicrons by intestinal enterocytes for extracellular transport, where such TAG-rich particles may end up ultimately contributing to serum levels of triacylglycerides in human beings.

[035] It is believed that the broadly-reactive lipase formulations of the invention deplete the 2-MAG component critical for the synthesis of new triacylglycerides by intestinal enterocytes which use the Monoacylglyceride Pathway. As a result, the lipase formulations of the invention reduce the levels of serum triacylglycerides.

Administration of Lipase Formulations

[036] The invention provides for administering an effective amount of the lipase formulations of the invention. The effective amount may include, for example, compositions comprising certain levels of lipase activity (measured in FIP units), or lipase amounts such that, when the lipase is administered for sufficient time period, serum TAG levels are reduced compared to serum TAG levels before administration the compositions.

[037] In one embodiment, the effective amount of the lipase comprises at least 15,000 FIP units per meal, which may be delivered by one or more capsules or tablets. For example, the effective amount may be at least 25,000, 40,000, 50,000, 70,000, 90,000, 100,000, 125,000, 150,000, 175,000, 200,000, 225,000, or 250,000 FIP units, which may be delivered by one or more capsules or tablets. It will be understood that the invention contemplates ranges of these effective amounts (e.g., 15,000 to 250,000 FIP units per meal, capsule or tablet).

[038] In another embodiment, the effective amount of the lipase comprises at least 2000 FIP units per gram of fat consumed by a human subject. For example, the enzyme's activity may be at least 2250, 2500, 3000, 3500, 4000, 4500, or 5000 FIP units per gram of fat consumed by a human subject. It will be understood that the invention contemplates ranges of these effective amounts (e.g., 2,000 to 5,000 FIP units per gram of fat consumed by a human subject).

[039] In another embodiment, the enzyme in the lipase composition of this invention comprises at least 180,000 FIP units per gram of the lipase. For example, the enzyme's activity may be at least 200,000, 225,000, 250,000, 300,000, 350,000, 375,000, 400,000, 450,000, or 500,000 FIP units per gram of the lipase. It will be understood that the invention contemplates ranges of these amounts (e.g., 180,000 to 500,000 FIP units per gram of the lipase).

[040] In another embodiment, the lipase may comprise at least 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, or 100% of the dosage weight. It will be understood that the invention contemplates ranges

of these amounts (e.g., the lipase may comprise 2 to 100% of the capsule or tablet weight). The remainder of the dosage may be any combination of known pharmaceutical excipents, so long as the excipients do not unduly degrade the stability of the lipase during storage.

[041] The lipase formulations of the invention are administered for a time period sufficient to reduce serum TAG levels compared to the serum TAG levels of the human subject to whom the lipase compositions of the invention had not been administered. For example, the lipase compositions may be administered over a time period sufficient to reduce serum triacylglycerides by at least 20, 25, 30, 35, 40, or 50% compared to the serum triacylglyceride level of the human subject before administering the composition to said human. In preferred embodiments, the period of chronic administration sufficient to reduce serum triacylglycerides by these levels may be for at least one, two, three, or four weeks (one month). In other embodiments, the compositions of the invention may be administered for longer periods of time such as two, four, six, nine, or twelve months. In yet another embodiment, the compositions of the invention may be administered as part of a daily regimen (e.g., administered with meals) for many years or the remainder of the subject's life.

[042] Typically, the lipase formulations of the invention are administered with food (e.g., with each meal) at the time the food (e.g., meal) is consumed. Alternatively, the lipase formulations may be administered "substantially contemporaneously with food." As used herein, "substantially contemporaneously with food" means that the lipase formulations are administered before or after ingestion of food (e.g., a meal) and preferably within 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 minutes before or after ingesting food (e.g., a meal).

[043] The lipase dosages of the invention are typically administered in the form of capsules or tablets. In a preferred embodiment, the lipase formulation is administered in a single capsule or

tablet. In another embodiment, the lipase formulation is administered using multiple capsules or tablets. Exemplary capsule or tablets may be 200-500mg.

Human Populations

[044] The invention provides for methods of administering the lipase formulations to human subjects having elevated TAG levels. In one embodiment, the human subject has above normal TAG levels (at least 150 mg/dL). In another embodiment, the human subject has borderline high TAG levels (150-199 mg/dL), high TAG levels (200-499 mg/dL), or very high TAG levels (≥500 mg/dL).

[045] The invention provides for methods of administering the lipase formulations to human subjects having diseases or conditions associated with elevated TAG levels. In one embodiment, the human subject is at risk for or has diabetes. In another embodiment, the human subject has diabetes and is compensating for low carbohydrate caloric intake with higher fat and protein intake. In another embodiment, the human subject is at risk for or has type 2 diabetes (e.g., the human subject has type 2 diabetes and is at risk for coronary disease).

Combinations of Various Embodiments and Concepts

[046] It will be understood that the embodiments and concepts described herein may be used in combination. For example, the invention provides for a method of reducing serum triacylglyercide levels in a human subject comprising administering a composition comprising an effective amount of a fungal lipase, wherein (i) the fungal lipase is from *C. cylindracea*; (ii) the effective amount of *C. rugosa* (*cylindracea*) is at least 15,000 FIP units/meal; (iii) the composition is administered over a time period sufficient to reduce serum triacylglycerides by at least 20% compared to the serum triacylglycerides of the human subject before administering the composition to said human; and (iv) said human subject has at least 150 mg/dL of serum triacylglycerides before administering the

composition. Accordingly, other combinations of lipases, effective amounts, modes of administrations, and patient populations are also envisioned.

Examples

[047] The following examples are not intended to limit the invention in any way.

Example 1 - Activity and Stable of Lipases

[048] Figures 4A and 4B show the effect of pH on the enzymatic activity (Figure 4A) and stability (Figure 4B) of *Candida rugosa* lipase. A Fungal Lipase International (FIP) activity assay (Food Chemical Codex protocol, Volume 7: 1201-1202, 2011) was used to determine that the *Candida rugosa* lipase is fully active in a pH range of 2-8, and preferably a pH range of 3-8. An FIP unit is the amount of enzyme required to release one micromole of fatty acid from triglyceride in one minute. The standard FIP assay uses an automatic pH titration instrument to maintain the neutral pH of the lipase reaction mixture (pH 7); the temperature is 37°C. As fatty acids are released by the lipase from the olive oil substrate, the pH drops and 0.02N NaOH solution is automatically added to the reaction mixture maintain the neutral pH. The measurement of the exact volume of the NaOH solution used to maintain a neutral pH during the reaction can be employed to calculate lipase activity.

[049] For the pH effects graph shown in Figure 4A, the automatic titration instrument was set to maintain the reaction at the different pH levels shown in the graph. The results indicate that *Candida rugosa* lipase is fully active at pH 3-4; a similar pH achieved by the nearly full stomach. The *Candida rugosa* lipase is stable at low pH and retains the majority of its enzymatic activity (Figure 4B). For these experiments, *Candida rugosa* lipase was pre-incubated in appropriate buffers at different pH levels for 2 hours at 37°C before performing the FIP activity assay (pH 7.0). Taken together, the data indicates that *Candida* lipase can begin breakdown of triacylglycerides in the full stomach.

[050] The effects of pH on the enzymatic activities and stabilities of two other microbial lipases are shown in Figures 5A-5D. The effect of pH on the enzymatic activity and stability of *Aspergillus niger* lipase is shown in Figures 5A and 5B, respectively. The lipase derived from *Aspergillus niger* demonstrated slightly less relative enzymatic activity at low pH compared to the *Candida rugosa* lipase, but shows similar consistent enzyme stability over a wide pH range. The effect of pH on the enzymatic activity and stability of *Rhizopus oryzae* lipase is shown in Figures 5C and 5D, respectively. The lipase derived from *Rhizopus oryzae* demonstrated significantly less relative enzymatic activity at low pH compared to the *Candida rugosa* lipase, and also shows less enzyme stability over a narrower pH range.

Digestion of Triacylglycerides Using Lipase Formulations (Examples 2-5)

[051] The inventors preformed hydrolysis experiments using various lipases at pH 4 and 7, and measured the amount of glycerol, if any, released from triacylglycerides by the lipases.

Hydrolysis Experiments

[052] The experiments tested the hydrolysis of triacylglyceride oils (soybean and olive oil) using the following lipases: pig pancreas lipase (American Labs Inc., Omaha NE), *Aspergillus niger* lipase (Bio-Cat Inc., Troy VA), *Rhizopus oryzae* lipase (Bio-Cat Inc.), and *Candida rugosa* lipase (Bio-Cat Inc.).

[053] A standard reaction protocol was used to assess triacylglyceride digestion by different lipases. The lipase reactions were performed using a 100ml jacketed beaker, kept at 37°C by using a Fisher Isotemp recirculating water bath. 5mL of 0.05M $CaCl_2$ and 1mL of 5mg/mL bile salts were added to the pre-warmed jacketed beaker. The reaction mixture beaker was slowly stirred using a stir bar on a magnetic stir plate. For most experiments (results shown in Figures 6, 7, and 9), 12,900 FIP units were used for hydrolysis (860 FIP Units per mL of oil). The lipases were weighed out on tarred weigh paper then transferred to a 20mL tube using 4mL water and vortexed until dissolved. The tube was rinsed twice with 4.25 mL of water, which was added to the reaction mixture. The

reaction was brought to pH 4.0 (or 7.0 in some experiments) by the addition of 0.02N NaOH. The lipase reaction was started by the addition of 15mL of soybean oil (figures 6-8; Food Lion, Troy, VA) or olive oil (figure 9; Sigma-Aldrich Chemicals, St. Louis, MO). Lipase reaction samples were obtained at 30, 60, 120 and 180 minutes and placed in microcentrifuge tubes. The samples were centrifuged for 5 minutes at 10,000rpm to separate the oil and water layers and then stored at -20°C in a freezer.

Triacylglyceride Digestion Analysis

[054] Triacylglyceride digestion was quantitated by HPLC. For HPLC analysis, the oil layer was dissolved in acetone, the amount dependent on sample concentration. The HPLC system consisted of an Agilent 1100 series Degasser, Quaternary Pump, Autosampler, Column Oven, Refractive Index Detector and two Supelcosil LC-18 Columns (150 x 4.6mm; Sigma-Aldrich) in series. The flow rate was 1 mL/min of Acetone/Acetonitrile (64:36, v/v) with the column oven set at 25°C. The Refractive Index Detector's Optical Temperature was set at 30°C and peak width was set at >0.2 min. The triglyceride results were quantitated by using a modified version of the separation methods employed by Perkins(1979) and Podlanta and Töregård(1982), and as described in Supelco Bulletin 787D (Sigma-Aldrich 1997).

Example 2 - Soybean Hydrolysis Results

[055] Figure 6 shows the results of the soybean oil hydrolysis at pH4. Compared to the other three lipases (n=4), *Candida* lipase (n=5) demonstrated significantly increased digestion of soybean oil triacylglycerides, at all four time points analyzed (*= $P<0.01$). Results were analyzed by a one-way ANOVA ($P<0.01$), followed by the post-hoc Tukey-Kramer HSD test, to determine significant differences between the lipase test groups. The standard error of the mean (SEM) bars are shown for the *Candida* lipase results. Similar results were found when the triacylglyceride reactions were performed at pH 7 (data not shown), except that pig pancreatic lipase showed a small amount of

lipase activity at pH 7. It is unknown whether the pig pancreatic lipase preparation used contained colipase, the potential absence of which may have contributed to the results observed.

Example 3 - Glycerol Analysis

[056] A comparative temporal digestion of soybean oil triglycerides by four lipases (each containing 12,900 FIP Units) was performed using the standard reaction protocol (pH 4). Samples were prepared by dilution of aqueous layer in water; the oil layer was extracted in water (50:50, v/v) by vortexing for 30 seconds. The oil/water mix was then centrifuged at 10,000 rpm for 5 minutes. The water layer was diluted as needed using water. 1 mg/mL glycerol stock solution and all standards were prepared in water.

[057] The amount of glycerol produced was quantitated by HPLC using a modified version of the separation method employed by Gandi *et al.* (The Application Notebook, Metrohm e-publication, LC_GC Chromatography online.com 12/02/2009). The HPLC system consisted of an Agilent 1100 series Degasser, Quaternary Pump, Autosampler, Column Oven, Refractive Index Detector and Supelocgel C-610H Carbohydrate Column (300 x 7.8 mm: Sigma-Aldrich)). The flow rate was 0.5 mL/min of 0.1% phosphoric acid with the column oven set at 30°C. The Refractive Index Detector's Optical Temperature was set at 30°C and peak width was set at >0.2 min.

[058] Figure 7 shows the results of the glycerol analysis. Compared to the other three lipases employed (n=4), *Candida* lipase (n=5) demonstrated significantly increased production of glycerol from complete digestion of soybean oil triglycerides at all four time points analyzed (*= $P<0.01$). Results were analyzed by a one-way ANOVA ($P<0.01$), followed by the post-hoc Tukey-Kramer HSD test, to determine significant differences between the lipase test groups. The standard error of the mean (SEM) bars are shown for the *Candida* lipase results. Similar results were found when the triacylglyceride reactions were performed at pH 7 and the amount of glycerol generated was

analyzed (data not shown), i.e., *Candida* lipase produced significantly more glycerol at both pH 4 and pH 7 than the other 4 lipases studied.

Example 4 - Concentration-Response Experiment

[059] Figure 8 shows concentration-response curves for *Candida* lipase. *Candida* lipase (500-12,900 FIP Units; 33.3-860 FIP Units per mL of oil) was reacted with soybean oil using the standard reaction protocol (pH 4) and the triacylglycerides were digested for 30 minutes. The amount of glycerol produced was quantitated by HPLC as described above. The amount of *Candida* lipase could be correlated with increased production of glycerol from complete digestion of soybean oil triglycerides, demonstrating the specificity of the reaction on glycerol production. The error bars depict the range of the amount of glycerol (duplicate to quadruplicate measurements) generated by the amount of *Candida* lipase used in the concentration response study.

Example 5 - Olive Oil and Shortening Experiments

[060] The inventors also tested whether the enhanced lipid digestive effects of *Candida* lipase on soybean oil triacylglycerides appears to be a general phenomenon applicable to action on other sources of triacylglycerides. As such, experiments were performed using olive oil and shortening as a source of triacylglycerides.

[061] Figure 9 shows the comparative actions of four lipases on triacylglyceride digestion and glycerol production using olive oil. A 30 minute digestion of olive oil triglycerides was made using each of the four lipases (each lipase reaction containing 12,900 FIP Units) using the standard reaction protocol (pH 4). The amount of triacylglycerides and glycerol produced by the lipase reactions were quantitated by HPLC using the methodologies described above.

[062] Control reactions (without lipase) contained 822 ± 2 mg/ml olive oil triacylglycerides and no free glycerol was detected.

[063] Similar results were found using olive oil as a source of triacylglycerides as when using soybean oil. Like with soybean oil, *Candida* lipase was the most effective lipase tested at digesting olive oil triacylglycerides and similarly produced the largest amount of glycerol during the digestive reaction. Similar results were found when using melted shortening (Crisco; Food Lion) dissolved in chloroform as a source of triacylglycerides (data not shown). *Candida* lipase effectively digested shortening triacylglycerides to the similar low levels seen with soybean and olive triacylglycerides at both pH 4 and pH 7 (109 mg/ml and 164 mg/ml remaining triacylglycerides detected, respectively, compared to control 856 mg/ml triacylglycerides). Thus, *Candida* lipase appeared to generally be the most effective enzyme tested to digest lipids.

Example 6 - Human Study Using Lipase Formulations

[064] A 1-month study was undertaken to determine the effects of a *Candida* lipase composition on serum triacylglyceride levels *in vivo*.

[065] Capsules containing 355 mg of the improved lipase composition were administered to three healthy human subjects. All of the components contained in the invention are GRAS (generally regarded as safe). The 355 mg capsules contained the following ingredients:

Capsule Component	Capsule Amount	Component Role
Candida rugosa lipase	238mg (100,000 FIP Units)	Active Ingredient
Magnesium Stearate	2mg	Lubricant, Anti-Sticking Agent
Microcrystalline Cellulose	96mg	Emulsifier, Anti-Caking Agent
Calcium Carbonate	19mg	Additive, Stabilizer

[066] The participants orally ingested a 355 mg capsule, during each meal, for a 1-month period. Serum levels of triacylglycerides were determined for each participant before the study began and

after the 1-month treatment. The serum levels of triacylglycerides and various other serum proteins were quantitated using local hospital laboratories certified as skilled in the performance of such tests.

[067] The results are shown in the following table:

Study Participant	Pre-Study TAG Classification	Pre-Study TAG Level (mg/dL)	Post-Study TAG Level (mg/dL)	Percent Reduction in TAG	Post-Study TAG Classification
A	High	428	236	45% Reduction	High
B	Borderline High	183	123	33% Reduction	Normal
C	Normal	92	91	1% Reduction	Normal

[068] The results indicate that *Candida* lipase was able to reduce serum levels of triacylglycerides in the two study participants who started the study with either borderline high or high levels of triacylglycerides. No consistent changes were noted in related parameters tested such as total cholesterol or its derivative (e.g., HDL) in the three study participants. The effect of *Candida* lipase treatment on the participant that started the study with normal serum levels of triacylglycerides was negligible.

[069] It is well-known that triacylglycerides can be produced and stored by adipose and liver cells. It is conceivable that there is a basal level of triacylglycerides in human beings produced by these alternative TAG synthetic sources that is influenced by genetics and environmental factors. As such, the compositions described herein may be best suited for individuals with borderline or high serum levels of triacylglycerides for whom dietary sources and enterocyte activities may contribute to higher serum levels of triacylglycerides.

[070] High serum levels of triacylglycerides (TAG) have been found in many patients that have type 2 diabetes (Bitzur *et al.*, Diabetes Care 32 (suppl 2): S373-S377, 2009). As such, reduction of

high serum levels of triacylglycerides by the compositions described herein may help reduce the risk of coronary disease in patients with type 2 diabetes.

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U.S. Publication Nos. 2001/0046493; 2003/0017144; and 2008/0279839.

AP van de Woestijne et. al. Obes. Rev. 10.1111 (epub ahead of print) July 12, 2011.

Claims

1. A method of reducing serum triacylglyceride levels in a human subject comprising, administering a composition comprising an effective amount of a fungal lipase that (i) exhibits at least 50% of its maximum activity over the pH range of 2.0 to 8.0; and (ii) cleaves all three ester sites of a triglyceride, to a human subject in need thereof, wherein the effective amount of said fungal lipase comprises at least 15,000 FIP units/meal.

2. The method of claim 1, wherein the fungal lipase comprises at least 180,000 FIP units per gram of protein.

3. The method of claim 1, wherein the effective amount of fungal lipase comprises at least 2000 FIP units per gram of fat consumed by said human subject.

4. The method of claim 1, wherein the fungal lipase is a lipase derived from the *Candida* family.

5. The method of claim 1, wherein the composition is administered periodically and over a time period sufficient to reduce serum triacylglycerides by at least 20% compared to the serum triacylglycerides of the human subject before administering the composition to said human.

6. The method of claim 5, wherein the composition is administered for at least one week.

7. The method of claim 1, wherein said human subject has at least 150 mg/dL of serum triacylglycerides.

8. A method of reducing serum triacylglyceride levels in a human subject comprising, administering a composition comprising an effective amount of a fungal lipase that (i) exhibits at least 50% of its maximum activity over the pH range of 2.0 to 8.0; and (ii) cleaves all three ester sites of a triglyceride, to a human subject in need thereof, wherein the composition is administered

periodically and over a time period sufficient to reduce serum triacylglycerides by at least 20% compared to the serum triacylglycerides of the human subject before administering the composition to said human.

9. The method of claim 8, wherein the fungal lipase comprises at least 180,000 FIP units per gram of protein.

10. The method of claim 8, wherein the effective amount of fungal lipase comprises at least 2000 FIP units per gram of fat consumed by said human subject.

11. The method of claim 8, wherein the fungal lipase comprises 50-100% of the composition's weight.

12. The method of claim 8, wherein the fungal lipase is a lipase derived from the *Candida* family.

13. The method of claim 8, wherein the composition is administered for at least one week.

14. The method of claim 8, wherein said human subject has at least 150 mg/dL of serum triacylglycerides.

15. A method of reducing serum triacylglyceride levels in a human subject comprising, administering a composition comprising an effective amount of a fungal lipase to reduce serum triacylglycerides in a human subject having at least 150 mg/dL of serum triacylglycerides, wherein said fungal lipase (i) exhibits at least 50% of its maximum activity over the pH range of 2.0 to 8.0; and (ii) cleaves all three ester sites of a triglyceride, to a human subject in need thereof.

16. The method of claim 15, wherein the fungal lipase comprises at least 180,000 FIP units per gram of protein.

17. The method of claim 15, wherein the effective amount of fungal lipase comprises at least 2000 FIP units per gram of fat consumed by said human subject.

18. The method of claim 15, wherein the fungal lipase is a lipase derived from the *Candida* family.

19. The method of claim 15, wherein the composition is administered for one week.

20. The method of claim 15, wherein said human subject is at risk for or has diabetes.

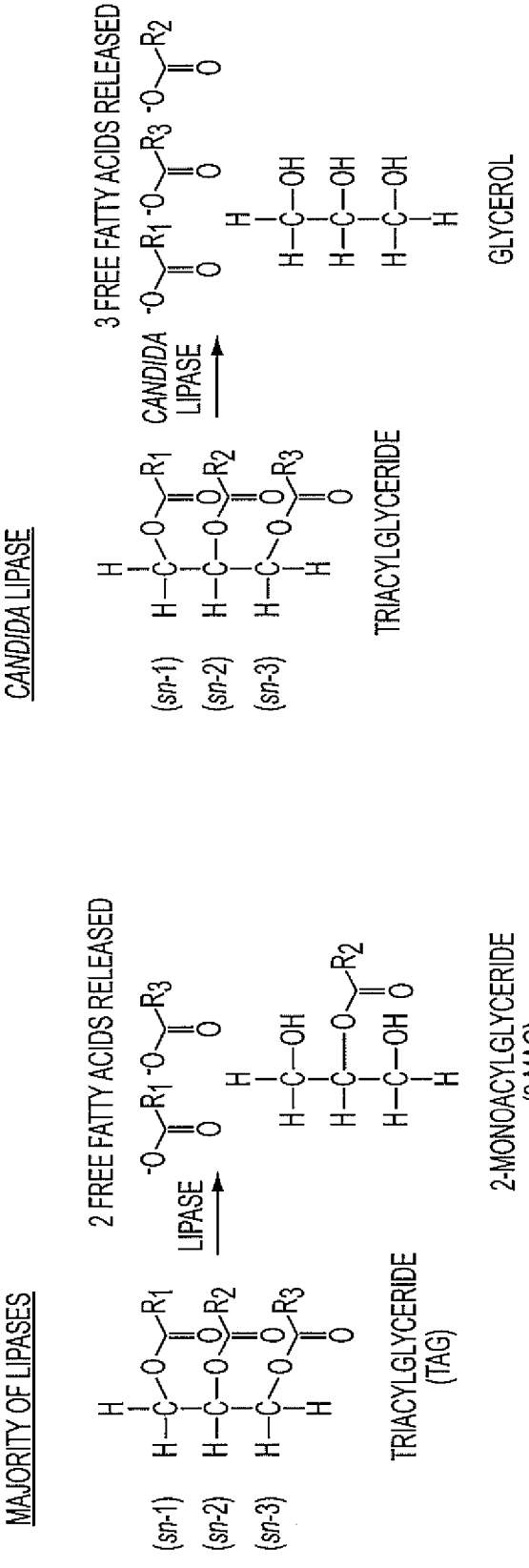

MAJORITY OF LIPASES
(sn-1)
(sn-2)
(sn-3)
LIPASE
2 FREE FATTY ACIDS RELEASED
TRIACYLGLYCERIDE
(TAG)
2-MONOACYLGLYCERIDE
(2-MAG)
CANDIDA LIPASE
CANDIDA
LIPASE
3 FREE FATTY ACIDS RELEASED
TRIACYLGLYCERIDE
GLYCEROL

FIG. 1

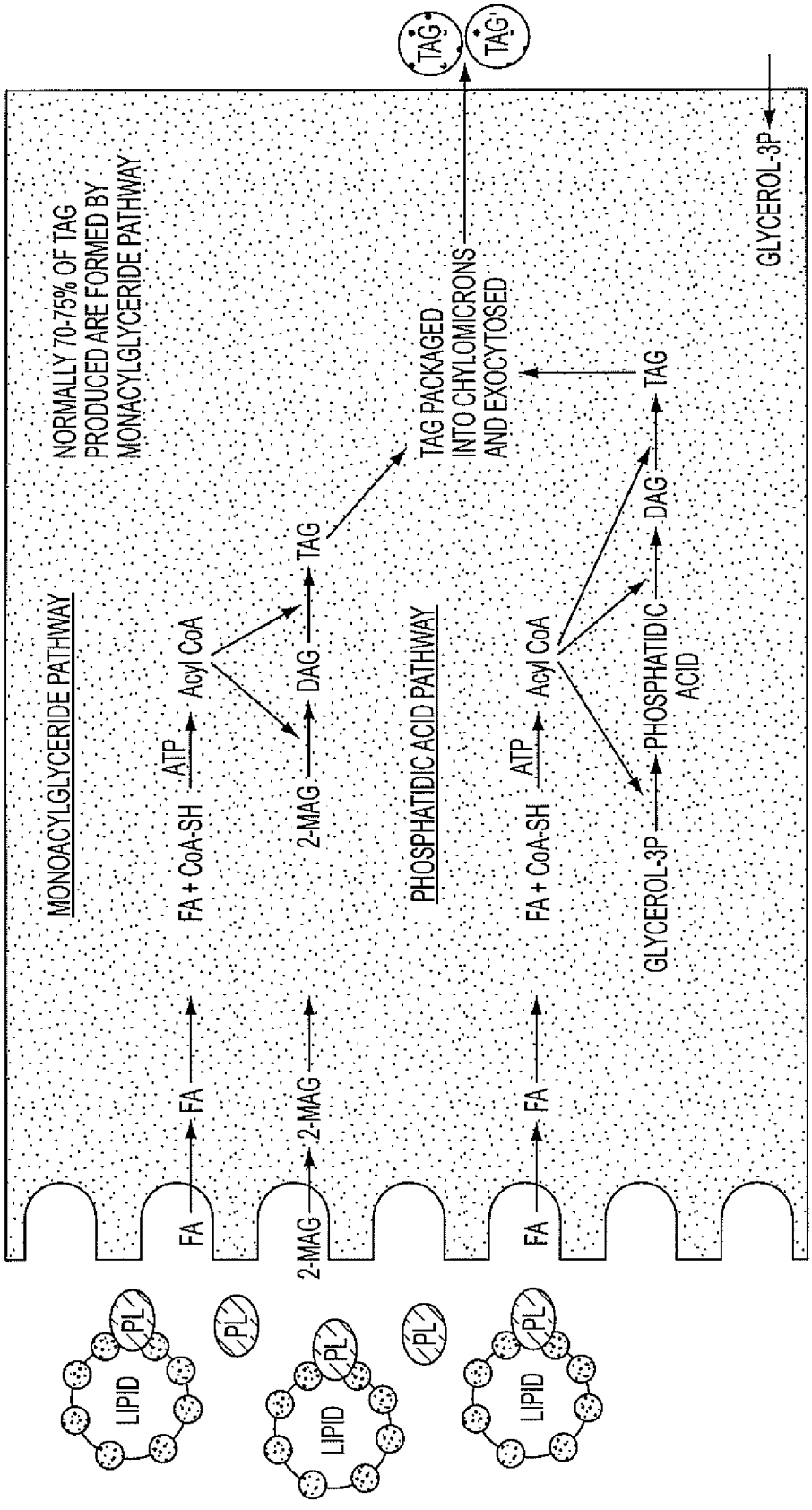

MONOACYLGLYCERIDE PATHWAY
NORMALLY 70-75% OF TAG PRODUCED ARE FORMED BY MONACYLGLYCERIDE PATHWAY
FA
FA + CoA-SH
ATP
Acyl CoA
2-MAG
DAG
TAG
TAG PACKAGED INTO CHYLOMICRONS AND EXOCYTOSED
PHOSPHATIDIC ACID PATHWAY
GLYCEROL-3P
PHOSPHATIDIC ACID
LIPID
PL

FIG. 2

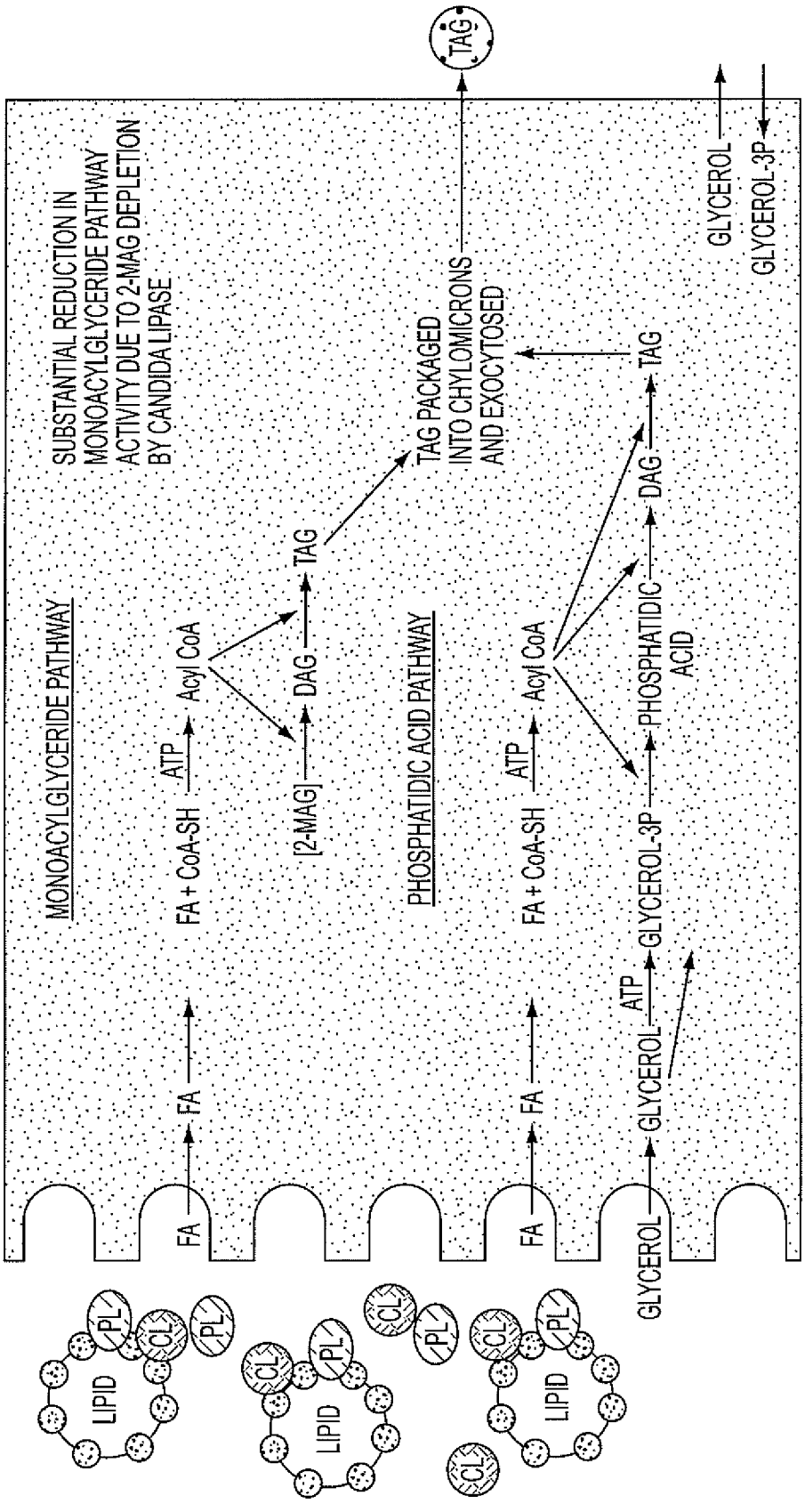

MONOACYLGLYCERIDE PATHWAY
SUBSTANTIAL REDUCTION IN MONOACYLGLYCERIDE PATHWAY ACTIVITY DUE TO 2-MAG DEPLETION BY CANDIDA LIPASE
FA
FA + CoA-SH
ATP
Acyl CoA
[2-MAG]
DAG
TAG
TAG PACKAGED INTO CHYLOMICRONS AND EXOCYTOSED
PHOSPHATIDIC ACID PATHWAY
GLYCEROL
ATP
GLYCEROL-3P
PHOSPHATIDIC ACID
GLYCEROL
GLYCEROL-3P
LIPID
PL
CL

FIG. 3

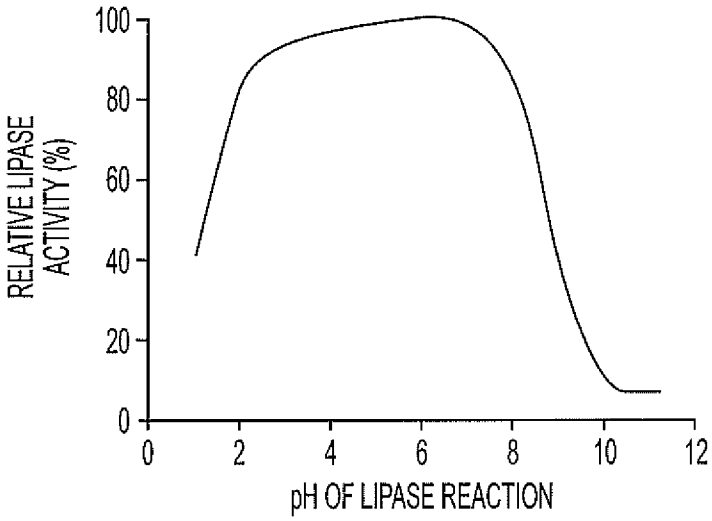
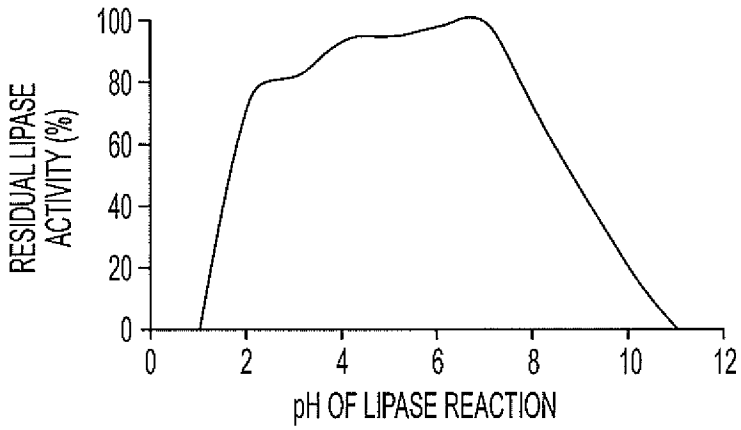

RELATIVE LIPASE ACTIVITY (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
pH OF LIPASE REACTION

FIG. 4A


RESIDUAL LIPASE ACTIVITY (%)
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60
40
20
0
0
2
4
6
8
10
12
pH OF LIPASE REACTION

FIG. 4B

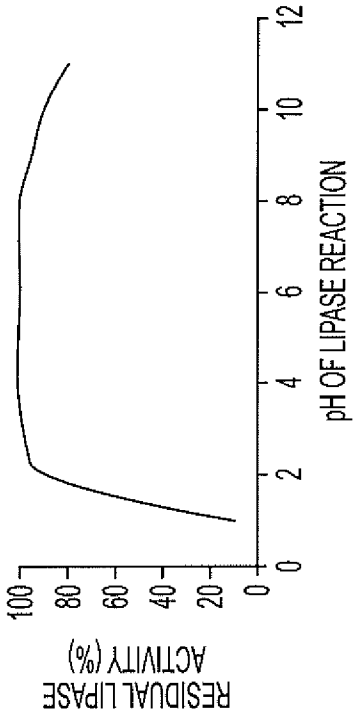

FIG. 5B

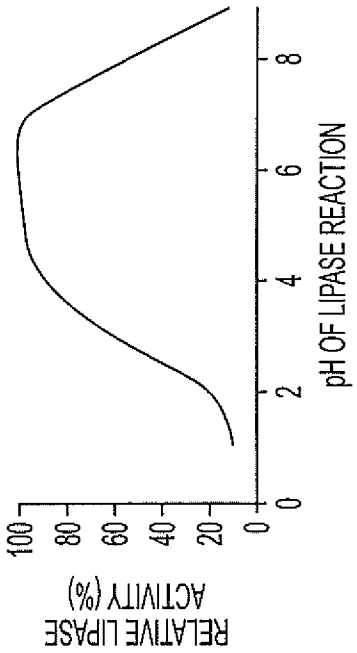

FIG. 5A

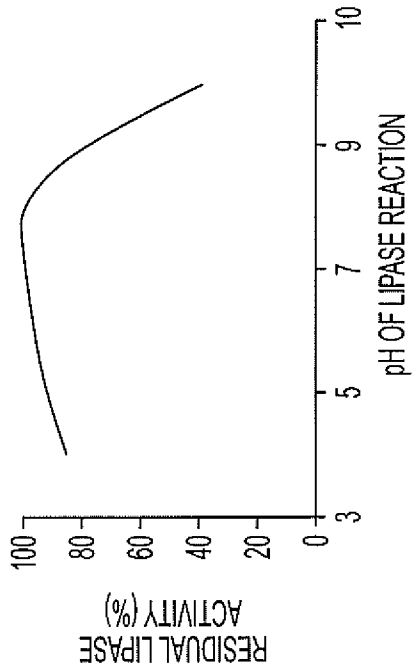

FIG. 5D

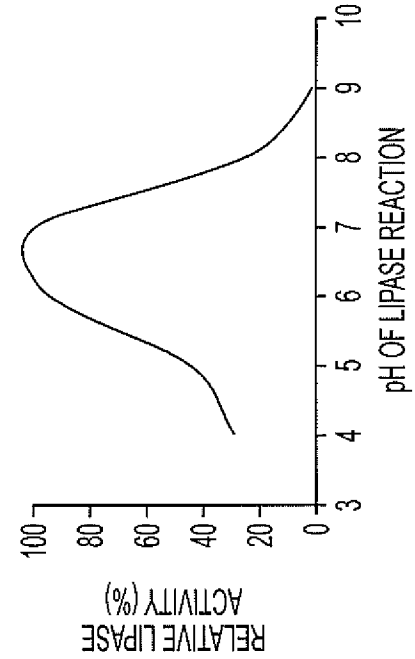

FIG. 5C

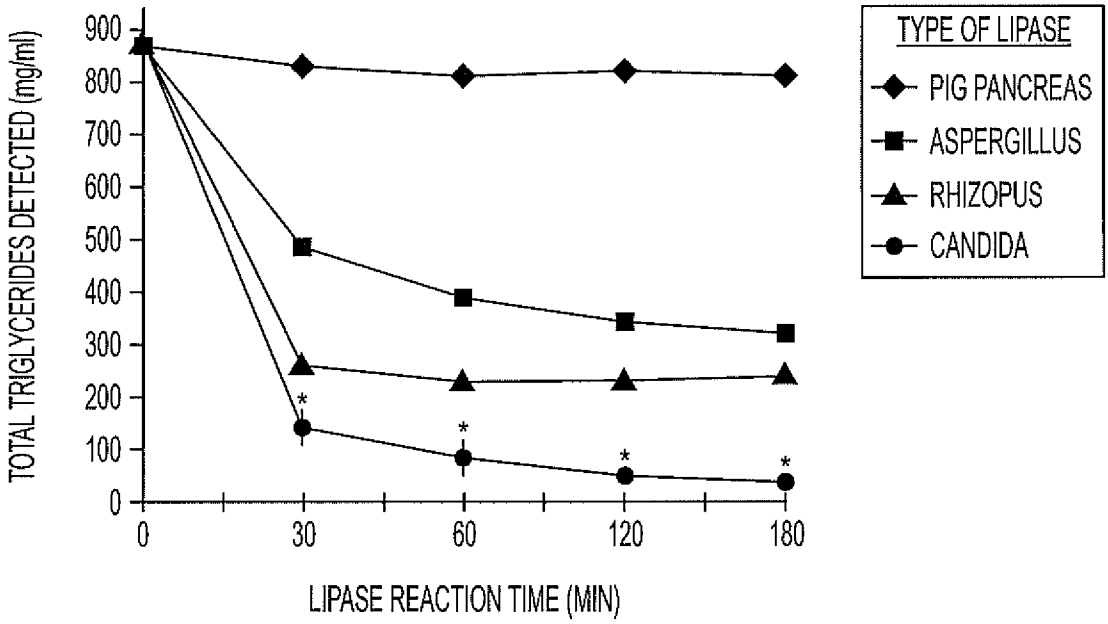
TOTAL TRIGLYCERIDES DETECTED (mg/ml)
900
800
700
600
500
400
300
200
100
0
0
30
60
120
180
LIPASE REACTION TIME (MIN)
TYPE OF LIPASE
PIG PANCREAS
ASPERGILLUS
RHIZOPUS
CANDIDA
*
*
*
*

FIG. 6

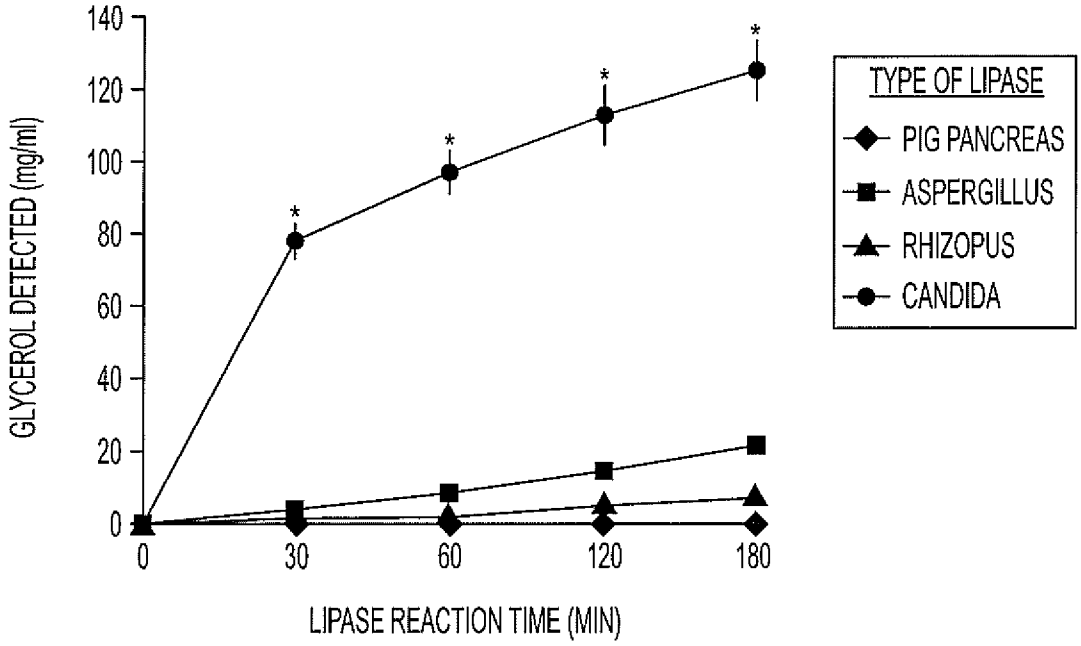
140
120
100
80
60
40
20
0
GLYCEROL DETECTED (mg/ml)
0
30
60
120
180
LIPASE REACTION TIME (MIN)
TYPE OF LIPASE
PIG PANCREAS
ASPERGILLUS
RHIZOPUS
CANDIDA

FIG. 7

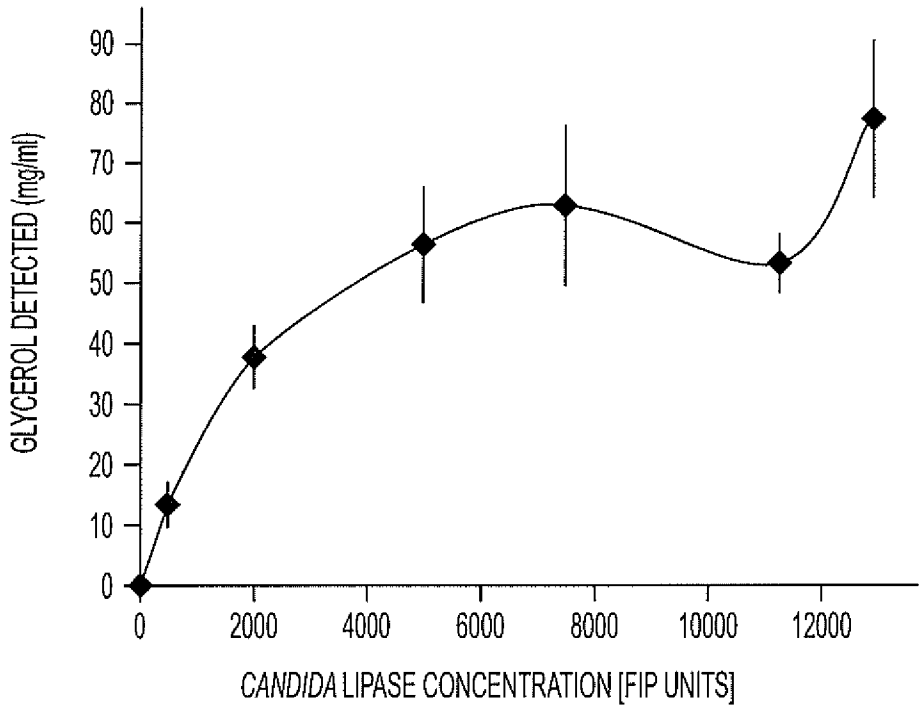
GLYCEROL DETECTED (mg/ml)
90
80
70
60
50
40
30
20
10
0
0
2000
4000
6000
8000
10000
12000
CANDIDA LIPASE CONCENTRATION [FIP UNITS]

FIG. 8

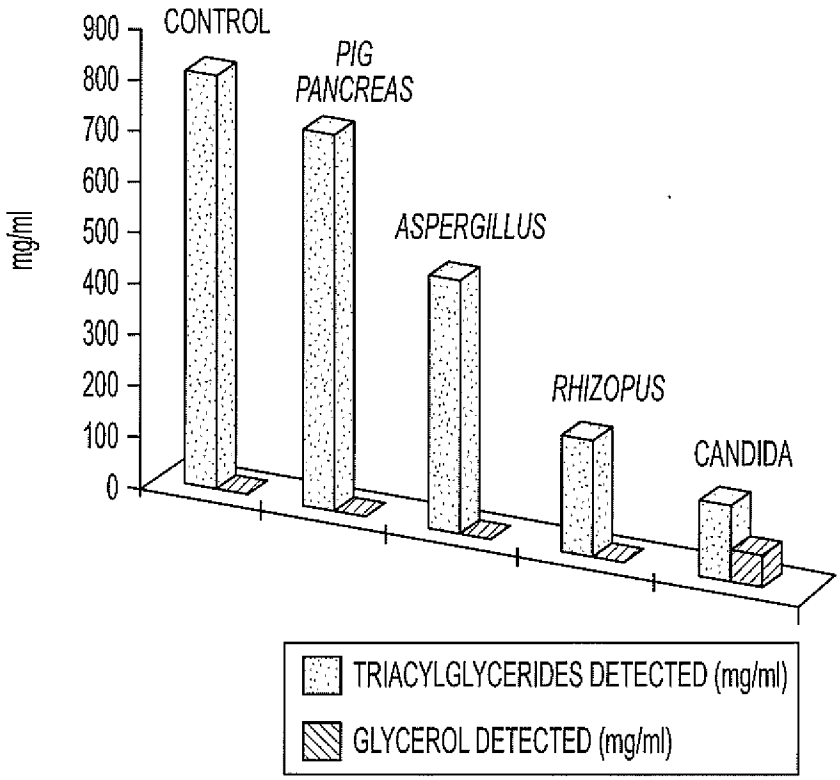
900
800
700
600
500
400
300
200
100
0
mg/ml
CONTROL
PIG PANCREAS
ASPERGILLUS
RHIZOPUS
CANDIDA
TRIACYLGLYCERIDES DETECTED (mg/ml)
GLYCEROL DETECTED (mg/ml)

FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US12/55899

A. CLASSIFICATION OF SUBJECT MATTER
IPC: **A61K 38/46**(2006.01)

USPC: 424/94.6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/94.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 6,699,496 B1 (KOJIMA et al.) 02 March 2004 (02.03.2004), see entire document.	1-6, 8-10, 12-13 ---------- 1-20
Y	US 2008/0279839 A1 (SCHULER et al.) 13 November 2008 (13.11.2008), see entire document.	1-20
Y	US 2010/0034797 A1 (SVENDSEN et al.) 11 February 2010 (11.02.2010), see entire document.	1-20
Y	HAFFNER(ed) "Dyslipidemia Management in Adults With diabetes", Diabetes Care, 2004, vol. 27, supp. 1, pgs. S68-S71.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 November 2012 (05.11.2012)	13 NOV 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer William Krynski Telephone No. 571-272-1700

Form PCT/ISA/210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT	International application No. PCT/US12/55899

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of:

   a. type of material

   ☐ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material

   ☐ on paper

   ☐ in electronic form

   c. time of filing/furnishing

   ☐ contained in the international application as filed

   ☐ filed together with the international application in electronic form

   ☐ furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT	International application No. PCT/US12/55899

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT	International application No. PCT/US12/55899

Box IV TEXT OF THE ABSTRACT (Continuation of Item 5 of the first sheet)

NEW ABSTRACT

INTERNATIONAL SEARCH REPORT	International application No. PCT/US12/55899

Continuation of B. FIELDS SEARCHED Item 3:
US-PGPUB; USPAT; EPO; DERWENT: schuler, christopher, edward, penet, moore, kenneth, gregory, kelly, candida, fungal, lipase, triacylglyceride, triglyceride, diabet