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(54) Titre : COMPOSITIONS PHARMACEUTIQUES ET PROCEDES DE REDUCTION DE LA GRAISSE CORPORELLE
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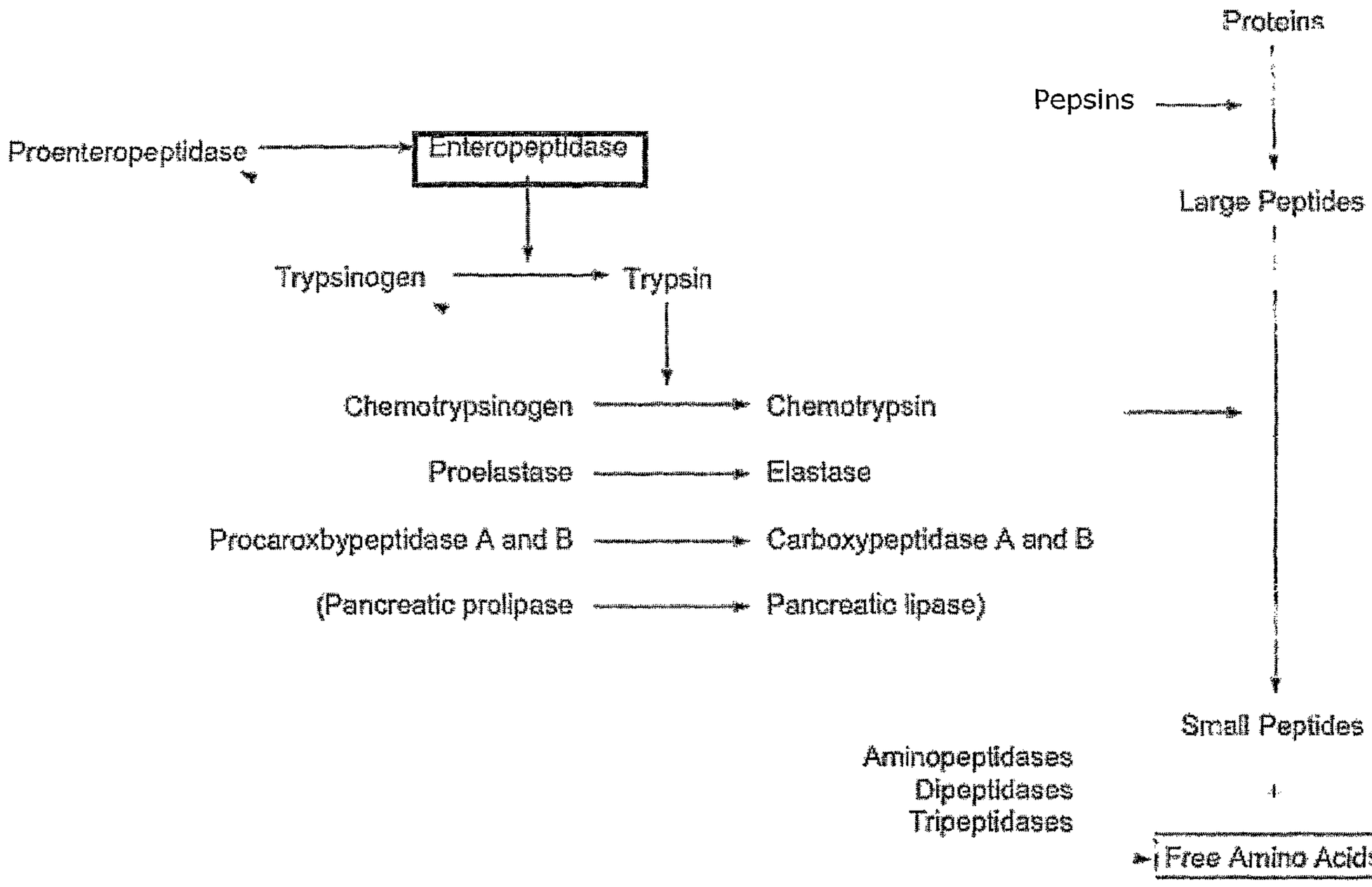

(57) Abrégé/Abstract:
The invention concerns a method of reducing body fat content of a subject in need thereof, the method comprising administering to the subject an agent capable of down-regulating activity and/or expression of at least one component participating in protein digestion and/or absorption. Such agents may be (i) an oligonucleotide directed to an endogenous nucleic acid sequence expressing said at least one component participating in said protein digestion and/or absorption or (ii) a protease inhibitor directed to said at least one component participating in protein digestion and/or absorption. The invention is particularly directed to a method of reducing body fat content of a subject in need thereof, the method comprising administering to the subject serine protease inhibitor inhibiting both enteropeptidase and trypsin activity.

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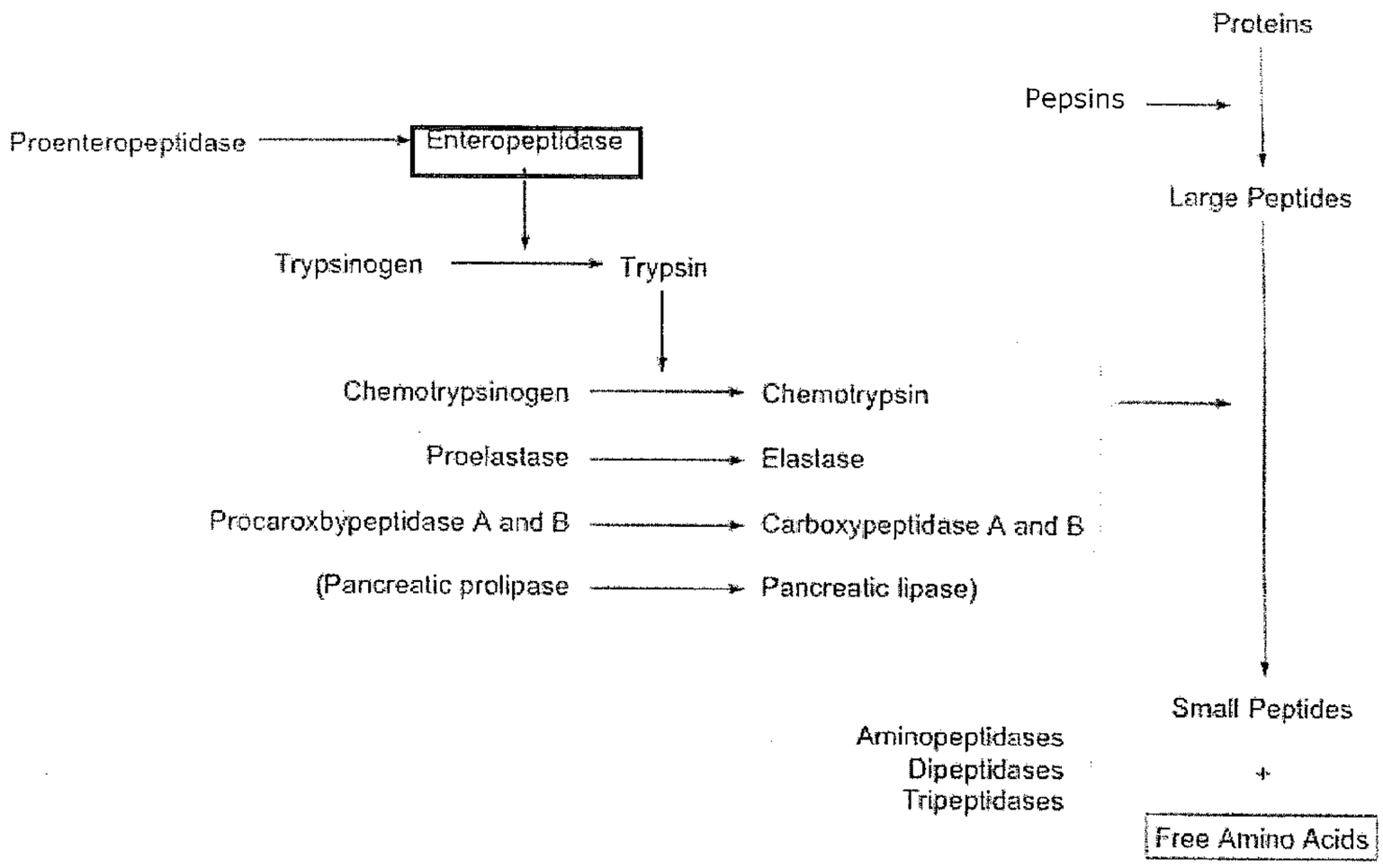

(57) Abstract: The invention concerns a method of reducing body fat content of a subject in need thereof, the method comprising administering to the subject an agent capable of down-regulating activity and/or expression of at least one component participating in protein digestion and/or absorption. Such agents may be (i) an oligonucleotide directed to an endogenous nucleic acid sequence expressing said at least one component participating in said protein digestion and/or absorption or (ii) a protease inhibitor directed to said at least one component participating in protein digestion and/or absorption. The invention is particularly directed to a method of reducing body fat content of a subject in need thereof, the method comprising administering to the subject serine protease inhibitor inhibiting both enteropeptidase and trypsin activity.

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CECI EST LE TOME	1	DE	2
CONTENANT LES PAGES	1	À	53

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THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME	1	OF	2
CONTAINING PAGES	1	TO	53

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PHARMACEUTICAL COMPOSITIONS AND METHODS OF REDUCING BODY FAT

The teachings of U.S. Provisional Patent Application No. 60/627,164, filed November 15, 2004, are hereby incorporated by reference.

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to pharmaceutical compositions and methods of reducing body fat.

Obesity is a multi-faceted chronic condition and is the most prevalent nutritional problem in the United States today. Obesity, a condition caused by an excess of energy intake as compared to energy expenditure, contributes to the pathogenesis of hypertension, type II or non-insulin dependent diabetes mellitus, hypercholesterolemia, hyperlipidemia, hypertriglyceridemia, heart disease, pancreatitis, and such common forms of cancer as breast cancer, prostate cancer, uterine cancer and colon cancer.

According to the 1999-2000 National Health and Nutrition Examination Survey (1999-2000 NAHNES, obesity and excessive weight affect more than 64 % of the U.S. adult population and it is predicted that obesity will become the primary cause of mortality by 2005. This phenomenon is not limited to the U.S. as increased numbers of obese individuals have recently been reported in Europe.

Obesity related genes have previously been described in the art as targets for the treatment of obesity. The obese gene (*ob*), which encodes for the circulating hormone leptin, and the diabetes gene (*db*), which encodes for its receptor [Tartaglia *et al.*, (1995) Cell 83(7): 1263-71; Zhang *et al.*, (1994) Nature 372(6505): 425-32], have both received wide attention. Leptin appears to regulate adipose tissue mass and also to modulate eating behavior. Although studies have shown that subcutaneous therapy with recombinant leptin can produce weight loss in both obese and lean subjects, it was found that most obese patients have high levels of endogenous leptin and are therefore leptin-resistant, a phenomenon that resembles insulin-resistance in diabetic patients [Considine *et al.*, (1996) N Engl J Med 334(5): 292-5; Maffei *et al.*, (1996) Diabetes 45(5): 679-82]. Thus, obese patients are mostly rendered insensitive to leptin (endogenous or exogenous). Additional examples of obesity related genes

include agouti (*ag*), tubby (*tub*), fat (*fat*), mahogany and neuropeptide Y (NPY) [Flier and Maratos-Flier (1998) Cell 92(4): 437-40; Spiegelman and Flier (1996) Cell 87(3): 377-89; Nagle *et al.*,(1999) Nature 398: 148-152; Gunn *et al.*, (1999) Nature, 398: 152-156], all of which are associated with satiety and appetite control by the central nervous system (CNS) and therefore have divergent physiological targets as well as affecting energy balance and obesity. In addition to these genes, it has been suggested that the mitochondrial uncoupling proteins (UCP) 1 and 2 , by preventing ATP synthesis and thus increasing glucose utilization, may also serve as targets for obesity treatment [Fleury *et al.*, (1997) Nat Genet 15(3): 269-72; Boss *et al.*, (1997) FEBS Lett 408: 39-42; Bouchard *et al.*, (1997) Hum. Mol. Genet. 11: 1887-1889]. However, all these proposed targets, as well as other obesity related genes, are highly limited by both their non-specificity and their redundancy, leading to associated substantial side effects [Nagle *et al.*, (1999) Nature 398: 148-152; Gunn *et al.*, (1999) Nature, 398: 152-156; Lu *et al.*, (1994) Nature 371: 799-802; Cool *et al.*, (1997) Cell 88: 73-83]. Furthermore, a lean phenotype has never been observed as a result of a deficiency of any of these gene products. Based on the "thrifty genome" theory, (which is described in detail by Neel [Am. J. Hum. Genet. (1962), 14, 353-362] and Coleman [Science(1979) 263, 663-665]), it was proposed that in most cases the genetic component of obesity involves a complex network of many genes creating various redundant biochemical pathways that stimulate appetite or satiety. Alternative inherited pathways therefore compensate for the inhibition or activation of a single pathway in order to maintain the same energy equilibrium.

At present, only a limited number of drugs for treating obesity are commercially available. Unfortunately, while some of these drugs may bring short-term relief to the patient, a long-term successful treatment has not been achieved as yet. Exemplary methods of treating obesity are also disclosed in U.S. Pat. Nos. 3,867,539; 4,446,138; 4,588,724; 4,745,122; 5,019,594; 5,300,298; 5,403,851; 5,567,714; 5,573,774; 5,578,613 and 5,900,411.

One of the presently available drugs for treating obesity, developed by Hoffman-LaRoche, is an inhibitor of pancreatic lipase (PL). Pancreatic lipase is responsible for the degradation of triglycerides to monoglycerides. However, it has been associated with side-effects such as severe diarrhea resulting in absorption inhibition of only one specific fraction of fatty acids and, has been known to induce

allergic reactions. Treatment with PL inhibitors is thus highly disadvantageous and may even expose the treated subject to life-threatening risks.

Recently, it has been suggested that fat absorption may be reduced by inhibiting the activity of the microsomal triglyceride-transfer protein (MTP), which is involved in the formation and secretion of very light density lipoproteins (VLDL) and chylomicrons. Sharp *et al.*, [Nature (1993) 365:65-69] and Wetterau *et al.*, [Science (1994) 282:751-754,] demonstrated that the mtp gene is responsible for abetalipoproteinemia disease. U.S. Pat. Nos. 6,066,650, 6,121,283 and 6,369,075 describe compositions that include MTP inhibitors, which are aimed at treating various conditions associated with excessive fat absorption. However, patients treated with MTP inhibitors suffer major side effects including hepatic steatosis, which are attributed to reduced MTP activity in both intestine and liver. This is not surprising since people naturally deficient for MTP activity were shown to develop fatty livers [Kane and Havel (1989); Disorders of the biogenesis and secretion of lipoproteins containing the apolipoprotein B. pp. 1139-1164 in: "The metabolic basis of inherited disease" (Scrivers *et al.*, eds.). McGraw-Hill, New York]. In fact, the company Brystol Myers Squibb, that developed MTP inhibitors for the treatment of obesity, has recently decided to abandon this target, due to this fatty liver side effect.

The presently known targets for the treatment of obesity and related disorders can be divided into four main classes: (i) appetite blockers, which include for example the NPY neuropeptide; (ii) satiety stimulators, which include, for example, the product of the *ob*, *db* and *agouti* genes; (iii) energy or fatty acid burning agents, which include the UCPs; and (iv) fat absorption inhibitors such as those acting on PL and MTP in the intestine, described above.

As is discussed hereinabove, the use of these targets is highly limited by their redundancy, their multiple targeting and/or their lack of tissue specificity.

There is thus a widely recognized need for, and it would be highly advantageous to have compositions and methods for treating obesity and related diseases and disorders devoid of the above limitations.

Energy is provided by carbohydrates (providing 25 % of the energy), fat (providing 50 % of the energy) and proteins (providing 25 % of the energy).

Protein metabolism strikes a balance between the body's energy and the synthetic needs and may contribute to the development of obesity. The four major

components of protein metabolism include protein synthesis, protein degradation, oxidation of amino acids and dietary intake of amino acids. When the dietary intake of protein is satisfactory, there is equilibrium between the various components of protein metabolism. Essentially, protein synthesis equilibrates with protein degradation. However, in many industrialized countries such as the United States, protein intake largely exceeds the needs of the individual. Thus, following mealtime, amino acid accumulation together with increased insulin, stimulates the storage of amino acids as protein. When the anabolic pathway is saturated, excess amino acids are oxidized. Oxidation products may either be used as substrates for energy production or may be converted to fat and stored in adipocytes, resulting in weight gain and ultimately contributing to the development of obesity.

On the other end of the scale, in times of starvation when glucose is scarce, gluconeogenesis occurs. Very little gluconeogenesis occurs in the brain, skeletal and heart muscles or other body tissues even though these organs have a high demand for glucose. Therefore, gluconeogenesis is constantly occurring in the liver to maintain the glucose level in the blood to meet these demands. However, in times of starvation, proteolytic degradation also plays a role in gluconeogenesis. Muscle releases lactate and glucogenic amino acids , that are converted to glucose in the liver via gluconeogenesis by direct entry into the citric acid cycle.

Protein metabolism provides 25 % of food energy. Excess dietary amino acids are oxidized and the end-products are used either to produce energy or converted to fat. The present inventors postulated that limiting dietary amino acid absorption (by inhibiting proteolytic degradation of proteins) can be used to treat obesity, since limiting amino acid absorption would ultimately result in reduction of body fat formation.

According to the ''thrifty genome'' theory, obesity genes may have conferred, in times of shortage of nutrition, some evolutionary advantages through efficient energy exploitation. Nevertheless, when food is abundant and way of life become sedentary, the same genes yield to obesity, type II diabetes and other obesity-related diseases. It is a challenge to identify crucial gene(s) in which mutations result in reduced energy intake. However ''expenditure genes'' or "lean genes" (as opposed to obesity genes) can also be considered as new potential targets for the treatment of obesity. These genes can be identified in rare genetic diseases with lean, failure to

thrive, malnutrition and/or energy malabsorption phenotype. For example, the congenital enteropeptidase deficiency, caused by mutations in the gene encoding the proenteropeptidase is characterized by a low body mass [A. Holzinger et al.; Am. J. Hum. Genet, 70:20-25; (2002)]. This pathology is usually successfully treated by pancreatic enzyme replacement or by dietary protein hydrolysate [Polonovski C, (1970). Arch. Franç. Péd 27:677-688]. A close pathology, the hydrochloric acid deficiency or achlorhydria, is also characterized by protein malabsorption and by a failure to thrive. In this pathology, the gastric pH is not acidic enough (above four). Pepsins are therefore not activated, and consequently ingested proteins are not digested into peptides. This ultimately leads to a considerably reduced intestinal digestion output.

Based on these observations correlating the EP deficiency or inactive pepsins with a thin phenotype and while searching for a novel therapeutic modality for obesity and related diseases, which would be devoid of the severe side effects known with the actually existing drugs, the present inventors postulated that pepsin activity, EP activity and/or underlying dietary enzymes activated thereby, may serve as selective and efficient targets for treating obesity.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods for reducing body fat of a subject.

It is another object of the present invention to provide compositions for treating a condition or disorder in which reducing body fat content is beneficial.

It is yet another object of the present invention to provide methods of treating a disease for which a low protein diet is beneficial in a subject.

Hence, according to the present invention there is provided a method of reducing body fat content of a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an agent capable of down-regulating activity and/or expression of at least one component participating in protein digestion and/or absorption, thereby reducing the body fat content of the subject.

According to another aspect of the present invention there is provided a method of reducing body fat content of a subject in need thereof, the method

comprising administering to the subject a therapeutically effective amount of an agent capable of down-regulating activity and/or expression of at least one component of an enteropeptidase pathway, thereby reducing the body fat content of the subject.

According to yet another aspect of the present invention there is provided a method of reducing a body fat content of a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an agent capable of down-regulating activity and/or expression of pepsin, thereby reducing the body fat content of the subject.

According to still another aspect of the present invention there is provided a pharmaceutical composition for treating a condition or disorder in which reducing body fat content is beneficial, comprising, as an active ingredient, a therapeutic effective amount of an agent capable of down-regulating activity and/or expression of at least one component participating in protein digestion and/or absorption and a pharmaceutically acceptable carrier.

According to an additional aspect of the present invention there is provided an article of manufacture comprising packaging material and a pharmaceutical composition identified for reducing body fat content of a subject in need thereof being contained within the packaging material, the pharmaceutical composition including as an active ingredient an agent capable of down-regulating activity and/or expression of at least one component participating in protein digestion and/or absorption pathway and a pharmaceutically acceptable carrier.

According to yet an additional aspect of the present invention there is provided a method of treating a disease for which low protein diet is beneficial in a subject in need thereof, the method comprising providing to the subject a therapeutically effective amount of an agent capable of down-regulating activity and/or expression of at least one component participating in protein digestion and/or absorption, thereby treating the disease for which low protein diet is beneficial in the subject in need thereof.

According to further features in preferred embodiments of the invention described below, the component participating in protein digestion and/or absorption is a protease, particularly a serine-protease or an aspartate-protease.

According to still further features in the described preferred embodiments, the protease is at least one component of an enteropeptidase pathway.

According to still further features in the described preferred embodiments, the at least one component of an enteropeptidase pathway is an activator of enteropeptidase.

According to still further features in the described preferred embodiments, the activator of enteropeptidase is duodenase.

According to still further features in the described preferred embodiments, the at least one component of an enteropeptidase pathway is enteropeptidase.

According to still further features in the described preferred embodiments, the at least one component of an enteropeptidase pathway is a downstream effector of enteropeptidase.

According to still further features in the described preferred embodiments, the downstream effector of enteropeptidase is selected from the group consisting of trypsin, chemotrypsin, elastase, carboxypeptidase A, carboxypeptidase B and pancreatic lipase.

According to still further features in the described preferred embodiments, the protease is a pepsin.

According to still further features in the described preferred embodiments, the pepsin is selected from the group consisting of Pepsin A, Pepsin B and Gastricin.

According to still further features in the described preferred embodiments, down-regulating activity and/or expression of at least one component participating in protein digestion and/or absorption is effected by an agent selected from the group consisting of: (i) an oligonucleotide directed to an endogenous nucleic acid sequence expressing at least one component participating in protein digestion and/or absorption; (ii) a protease inhibitor directed to at least one component participating in protein digestion and/or absorption.

According to still further features in the described preferred embodiments, the protease inhibitor is an aspartic protease inhibitor.

According to still further features in the described preferred embodiments, the aspartic protease inhibitor is a peptidomimetic aspartic protease inhibitor.

According to still further features in the described preferred embodiments, the peptidomimetic aspartic protease inhibitor is selected from the group consisting of CGP53437, Amprenavir, Atazanavir, Indinavir, Lopinavir, Fosamprenavir, Nelfinavir, Ritonavir and Saquinavir.

According to still further features in the described preferred embodiments, the aspartic protease inhibitor is a low molecular weight aspartic protease inhibitor.

According to still further features in the described preferred embodiments, the low molecular weight aspartic protease inhibitor is pepstatin.

According to still further features in the described preferred embodiments, the aspartic protease inhibitor is extracted from a plant.

According to still further features in the described preferred embodiments, the plant is selected from the group consisting of *Solanum tuberosum* (potato), *Cucurbita maxima* (squash) and *Anchusa strigosa* (Prickly Alkanet).

According to still further features in the described preferred embodiments, the aspartic protease inhibitor is extracted from a parasite.

According to still further features in the described preferred embodiments, the parasite is selected from the group consisting of *Ascaris suum* and *Ascaris lombricoides*.

According to still further features in the described preferred embodiments, the aspartic protease inhibitor is pepsine inhibitor-3 (PI-3).

According to still further features in the described preferred embodiments, the protease inhibitor is a serine protease inhibitor.

According to still further features in the described preferred embodiments, the serine protease inhibitor is a low molecular weight serine protease inhibitor.

According to still further features in the described preferred embodiments, the serine protease inhibitor is a peptidomimetic serine protease inhibitor.

According to still further features in the described preferred embodiments, the agent is linked to a mucoadhesive agent.

According to still further features in the described preferred embodiments, the mucoadhesive agent is a mucoadhesive polymer.

According to still further features in the described preferred embodiments, the mucoadhesive polymer is selected from the group consisting of chitosan, polyacrylic acid, hydroxyprpyl methylcellulose and hyaluronic acid.

According to still further features in the described preferred embodiments, the subject in need thereof is afflicted with a condition or disorder selected from the group consisting of excessive weight, obesity, type II diabetes, hypercholesterolemia, atherosclerosis, hypertension, pancreatitis, hypertriglyceridemia and hyperlipidemia.

According to still further features in the described preferred embodiments, the administering to the subject is effected by oral administration.

The present invention successfully addresses the shortcomings of the presently known configurations by providing methods of reducing body fat content.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more details than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

Figure 1 is a scheme illustrating components of the initial pepsin digestion of dietary proteins (right) and of the enteropeptidase activation cascade (left).

Figure 2 is the nucleic sequence and corresponding amino acid sequence of the human enteropeptidase (PRSS7)

The first line indicates the nucleotide sequence, grouped by codons; the second line indicates the amino acid sequence corresponding to the above codons with the three-letter code. The first codon of translation is shown in bold as well as the stop

codon. Numbering of the nucleic acids is at the right end of the first line, whereas numbering of the amino acids is indicated under amino acid residue (third line).

Figure 3 is the nucleic sequence and corresponding amino acid sequence of the human trypsin (PRSS1)

The first line indicates the nucleotide sequence, grouped by codons; the second line indicates the amino acid sequence corresponding to the above codons with the three-letter code. The first codon of translation is shown in bold as well as the stop codon. Numbering of the nucleic acids is at the right end of the first line, whereas numbering of the amino acids is indicated under amino acid residue (third line).

Figure 4 is the acidic propeptide of tryspinogen. The vertical arrow shows the site of cleavage of the tryspsinogen by the enteropeptidase, between the Lys (P1) and the Ile, releasing the activation peptide (left part) and the active form of trypsin (right part).

Figure 5 is a scheme of the trypsinogen activation assay. The release of pNA (p-nitroaniline) is measured as the result of the successful cleavage of the substrate N-CBZ-Gly-Pro-Arg-pNA by trypsin, which activity is the result of the cleavage of the tryspinogen by enteropeptidase.

Figure 6 is IC50 measurements calculated by the trypsinogen activation assay. The graphs represent the percentage of inhibition (as compared to a value without inhibitor) in function of various concentrations of inhibitors, i.e. AC-Leu-Val-Lys-Aldhehyde (**A**), H-D-Tyr-Pro-Arg-chloromethylketone trifluroroacetate salt (**B**) and Z-Asp-Glu-Val-Asp-chloromethylketone (**C**).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of pharmaceutical compositions and methods of reducing body fat content.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in details, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to

be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Excessive weight and obesity are widely recognized health problems, with approximately 97 million people considered clinically overweight or obese in the United States alone. These two conditions are associated with a number of psychological and medical ailments including atherosclerosis, hypertension, type II or non-insulin dependent diabetes mellitus, pancreatitis, hypercholesterolemia and hyperlipidemia.

Obesity results from greater energy intake than energy expenditure. Thus, treatment of obesity seeks to re-address this balance so that energy input is reduced below energy expenditure.

While conceiving the present invention, the inventors postulated that limiting protein digestion and/or absorption can be used as a method for reducing body fat content, and as such for treating obesity and related diseases.

Energy is provided by the ingestion of carbohydrates (providing 25 % of the energy), fat (providing 50 % of the energy) and proteins (providing 25 % of the energy).

Glucose is the metabolite of choice of both brain and working muscle. It cannot be synthesized from fatty acids because neither pyruvate nor oxaloacetate, the precursors of glucose in gluconeogenesis, can be synthesized from acetyl-CoA. During starvation, glucose must therefore be synthesized from amino acids derived from the proteolytic degradation of proteins, the major source of which is muscle, resulting in loss of muscular mass.

Protein metabolism strikes a balance between the body's energy and the synthetic needs and contributes to the development of obesity. The four major components of protein metabolism are protein synthesis, protein degradation, amino acid oxidation and dietary intake of amino acids. When the dietary intake of protein is satisfactory, there is an equilibrium between the various components of protein metabolism. Essentially, protein synthesis equilibrates with protein degradation. However, in many industrialized countries such as the United States, protein intake largely exceeds the needs of the individual. Thus, following mealtime, amino acid intake together with increased insulin, stimulates the storage of amino acids as protein. When the anabolic pathway is saturated, excess amino acids are oxidized.

The subsequent oxidation products are either used to produce energy or are converted to fat and stored in adipocytes, resulting in weight gain and ultimately contributing to the development of obesity.

Therefore, the limiting of excess amino acid absorption by the inhibition of protein degrading enzymes should assist in the prevention of body fat accumulation. Furthermore, it is believed that limiting excess amino acid absorption does not prohibit the body from metabolizing the continued supplies of fat and carbohydrates. However, since these sources are insufficient to compensate for the energy loss resulting from poor amino acid absorption, depletion in fat and carbohydrate (i.e. glycogen) stores should occur [Guyton and Hall "The Textbook of Medical Physiology" 10th Ed. Harcourt International Edition].

Thus, the present invention can be successfully used for reducing body fat content in an individual. As an illustration, an individual consuming 2000 kcal/day and burning 1800 kcal will have an excess 200 kcal which, in turn, will be transformed to fatty acids and stored as fat, thereby gaining weight. Treating such an individual with agents of the present invention (further described herein below) at a concentration which would limit protein digestion and/or absorption and reduce the number of calories assimilated to less than those expended would enable weight loss. Since 25 % of energy is attributed to protein metabolism a maximum number of 500 calories can be prevented from being assimilated by the present invention. However, since there is an amount of protein and corresponding amino acids that is essential to the body, measures are taken such that only a proportion of proteins is not digested and/or absorbed and thus not the total 500 calories should be prevented from being assimilated but a proportion thereof, as further described hereinbelow.

Thus, according to one aspect of the present invention there is provided a method of reducing body fat content of an individual subject.

As used herein the phrase "reducing body fat content" refers to reducing levels of mobilizable fat (e.g., fat contained in the blood) and fat tissue, which contains stored fat (e.g., adipose tissue).

As used herein the term "fat" refers to glycerol esters of saturated fatty acids such as triglycerides and fat-like substances such as steroid alcohols such as cholesterol.

The method, according to this aspect of the present invention is effected by providing to a subject in need thereof (e.g., an obese individual) a therapeutically effective amount of an agent capable of down-regulating activity and/or expression of at least one component participating in protein digestion and/ or absorption, thereby limiting body fat storage and, therefore enhancing fat catabolism in fat cells of the subject thereby reducing the body fat mass of the subject.

The phrase "fat catabolism" refers to the process of breaking down ingested and stocked fat into fatty acids and glycerol and subsequently into simpler compounds that can be used by the body as a source of energy.

As used herein, the phrase "subject in need thereof" refers to a mammal, preferably a human, which can benefit from enhancing its fat catabolism using the agents of the present invention. Examples are human subjects or domestic animals (e.g., cats, dogs, cattle, sheep, pigs, goats, poultry and equines) that suffer from the diseases or conditions listed hereinbelow.

As used herein, the phrase "protein digestion" refers to the process by which proteins are broken down into peptides and amino acids. This process is effected in both the stomach and the small intestine (Figure 1). Components which participate in protein digestion include proteolytic enzymes (i.e., proteases) and agents thereof including co-factors which are responsible for their activation.

As used herein, the phrase "protein absorption" refers to the process of amino acid and peptide absorption. This process is effected in the small intestine. Components, which participate in amino acid absorption, include amino acid receptors and transporters (e.g., sodium dependent amino acid transporters).

Preferably, in a first embodiment, the method of the invention is effected by down-regulating the expression and/or the activity of a protease that participates in protein digestion and/or absorption.

As used herein a "protease" refers to an enzyme that cleaves peptide bonds, which link amino acids together in protein molecules. Proteases comprise two groups of enzymes: (1) the endopeptidases that cleave peptide bonds within the protein and (2) the exopeptidases, which cleave peptide bonds removing amino acids sequentially from either the N or the C-terminus, respectively.

Preferably, in a first embodiment, the method of the present invention is effected by down-regulating the stomach enzyme, pepsin, which is active in the first

step of protein digestion, breaking down proteins into large peptides. Pepsin is the active form of its inactive precursor pepsinogen (i.e., zymogen) where the acid environment of the stomach triggers its activation. Protein chains bind in the deep active site groove of pepsin, and are degraded into large peptides, which are later degraded into small peptides by intestinal enzymes. It is suggested that blockade of the first step of protein digestion would reduce further protein absorption in the intestine. Noteworthy, hydrochloric acid deficiency or achlorhydria, is characterized by protein malabsorption and by a failure to thrive. In this pathology, the gastric pH is not acidic enough (above four) to convert pepsinogen to pepsin. Consequently ingested proteins are not digested into peptides. This ultimately leads to a considerably reduced intestinal digestion output.

The pepsin family has three members, Pepsin A, Pepsin B and Gastricin, all of which belong to the aspartic protease family. They are all expressed in the stomach and are the first proteolytic enzymes of the gastrointestinal digestive system [See Figure 1]. These enzymes are responsible for the break-down of proteins into large peptides. As these three enzymes are very similar, they are usually referred to indistinctly as Pepsins.

The aspartic protease family exists in vertebrates, plants and viruses. It includes Pepsins, the Cathepsin D, the Angiotesin-Converting Enzyme, the β-secretase and the HIV protease. They are characterized by the highly conserved sequence of Asp-Thr-Gly and are, with the exception of HIV protease which is a dimer of two identical subunits, monomeric enzymes comprising two domains. In general, aspartic proteases are highly specific cleaving peptide bonds between hydrophobic residues as well as a beta-methylene group. Pepsins, however, are considered to be proteases with broad structural specificity; an essential characteristic for their role in digestion. They do however elicit a preference for aromatic amino acid residues like phenylalanine.As used herein, pepsin refers to an aspartic protease of the pepsin family [e.g., Pepsin A (e.g., EC 3.4.23.1), Pepsin B (e.g., EC 3.4.23.2) and Gastricin eg. (EC 3.4.23.3) and to zymogens thereof such as, for example, Pepsinogen A (e.g., EC 3.4.23.1) Pepsinogen B (e.g., EC 3.4.23.2) and Progastricin.

As mentioned, large peptides generated by the action of pepsin, are broken down further in the intestine into smaller peptides and free amino acids by proteases of the enteropeptidase pathway (see Figure 1).

Thus, according to a second embodiment of the present invention, the method is effected by down-regulating at least one component of the enteropeptidase pathway (i.e., activators of enteropeptidase, enteropeptidase itself and downstream effectors of enteropeptidase, e.g., see Figure 1), which governs intestinal protein degradation and pancreatic lipase activation, thereby allowing inhibition of energy absorption deriving from proteins and from triglycerides.

As used herein "enteropeptidase" refers to a heterodimeric serine protease that activates trypsins and downstream proteases (e.g., EC 3.4.21.9). The serine protease enteropeptidase (EP, also termed enterokinase) is present in the duodenal and jejunal mucosa and is involved in the second phase of digestion of dietary proteins. Specifically, EP catalyzes the conversion, in the duodenal lumen, of trypsinogen into active trypsin *via* the cleavage of the acidic propeptide from trypsinogen. The activation of trypsin initiates a cascade of proteolytic reactions leading to the activation of many pancreatic zymogens. [See Figure 1 and Antonowicz, Ciba Found. Symp., 70: 169-187 (1979); Kitamoto *et al.*, Proc. Natl. Acad. Sci. USA, 91(16): 7588-7592 (1994)]. EP is highly specific for the substrate sequence $(Asp)_4$-Lys-Ile present in the trypsinogen molecule, where it acts to mediate cleavage of the Lys-Ile bond (Figure 4).

Enteropeptidase is a disulfide-linked heterodimer composed of a heavy chain of 82-140 kDa, and a light chain of 35-62 kDa [Mann (1994) Proc. Soc. Exp. Biol. Med. 206:114-8]. Mammalian EPs contain 30-50 % carbohydrates, which may contribute to the apparent differences in its peptide weight. The heavy chain is postulated to mediate association with the intestinal brush border membrane [Fonseca (1983) J. Biol. Chem. 258:14516-14520], while the light chain contains the catalytic site localized in the intestine lumen. Nucleotide and protein Accession numbers (according to NCBI) of enteropeptidase from different organisms are given in Table 1.

Table 1

Organism	Nucleotide	Protein	Protein size (in amino acid)
H. sapiens	NM_002772	NP_002763	1019
P. troglodytes	XM_514836	XP_514836	1089
C. familiaris	XM_544824	XP_544824	1034
M. musculus	NM_008941	NP_032967	1069
R. norvegicus	XM_213668	XP_213668	1042
B. taurus	NM_174439	NP_776864	1035
S. scrofa	NM_001001259	NP_001001259	1034
G. gallus	XM_425539	XP_425539	1044

As used herein a "downstream effector" refers to a target molecule in a pathway. The downstream effectors of enteropeptidase include the serine proteases trypsins (e.g., EC 3.4.21.4), chymotrypsin (e.g., EC 3.4,21.1), elastases (e.g., EC 3.4.21.36), and the metalloproteases carboxypeptidase A, carboxypeptidase B and pancreatic lipase and zymogens thereof, as well as, enzymes participating in the hydrolysis of small peptides such as aminopeptidases (e.g., EC 3.4.11.2), dipeptidases (e.g., EC 3.4.13.18) and tripeptidases (EC 3.4.11.4). Nucleotide and protein Accession numbers of tryspin from different organisms are given in Table 2.

Table 2

Organism	Nucleotide	Protein	Protein size (in amino acid)
H. sapiens	NM_002769	NP_002760	247
P. troglodytes	XM_519441	XP_519441	247
C. familiaris	XM_532744	XP_532744	246
M. musculus	NM_053243	NP_444473	246
R. norvegicus	NM_012729	NP_036861	246
B. taurus	NM_174690	NP_777115	247
G. gallus	AAN75630	AF110982	248

An example of an activator of enteropeptidase is the serine protease, duodenase [Zamolodchikova *et al.*, 1995 Eur J Biochem 227, 866-872]. Since

duodenase and enteropeptidase control this important protein digestive pathway in addition to the pancreatic lipase activity, agents which are directed at either or both of these targets are currently preferred according to this aspect of the present invention, to avoid redundancy.

Agents capable of down-regulating activity or expression of proteins or mRNA transcripts encoding thereof are well known in the art.

Since many of the protein targets of the present invention are localized in the lumen of the small intestine, which is featured by high protease activity, agents of the present invention (e.g., protein agents) are preferably modified to increase bioavailability thereof. Thus, agents of the present invention may be chemically modified to improve their stability. Agents of the present invention may be administered using bioadhesive delivery systems capable of enhancing contact of the drug with the mucous membrane lining the gastro-intestinal tract. Furthermore, the use of carrier systems such as micro-spheres and nanoparticles that can improve the bioavailability of the agents may be preferred [see Pappas (2004) Expert Opin. Biol. Ther. 4:881-7; Cefalu (2004) Drugs 64:1149-61; and Gowthamarajan and Kulkarni (2003) Resonance 38-46].

For example, agents of the present invention, including protease inhibitors, oligonucleotides, antibodies, antibody fragments and non functional derivatives of the components of the pathways discussed herein are preferably combined with a mucoadhesive agent in order to improve drug delivery. Various mucoadhesive agents, e.g., mucoadhesive polymers are known which are believed to bind to the mucus layers coating the stomach and other regions of the gastrointestinal tract. Examples of mucoadhesive polymers as discussed herein include, but are not limited to chitosan, polyacrylic acid, hydroxypropyl methylcellulose and hyaluronic acid. Most preferably, the mucoadhesive polymer is chitosan [Guggi *et al.*, (2003) J of Controlled Release 92:125-135].

In one preferred embodiment, the agent is a protease inhibitor, which is designed to specifically inhibit the activity or the expression of a particular protease participating in protein digestion and/or absorption (see above). For example, when the protease target is of the enteropeptidase pathway, a serine protease inhibitor is preferably used. Particularly interesting are protease inhibitor having a cumulative effect on both enteropeptidase and trypsin *i.e.*, agents that are able to inhibit both

enteropeptidase and trypsin activities. Also concerned are protease inhibitors having an inhibitory effect on enteropeptidase or trypsin only. When the down-regulated protease is pepsin, an aspartic protease inhibitor is required. A synthetic protease inhibitor, such as camostat, may also be used.

Aspartic protease inhibitors which can be utilized by the present invention are well known in the art. Examples include, but are not limited to, naturally occurring or synthetic, low or high molecular weight inhibitors including peptide or non-peptide based inhibitors. As used herein, a low molecular weight inhibitor is one which is typically below one kilodalton.

Aspartic protease inhibitors which can be utilized by the present invention to inhibit pepsin include, but are not limited to, the high molecular weight synthetic peptidomimetic protease inhibitors. The mechanism of action of these peptide-based inhibitors involves the formation of a transition-state analogue. Examples of peptidomimetic protease inhibitors of pepsin include retroviral protease inhibitors which are typically utilized in the treatment of human immunodeficiency virus (HIV) and hepatitis C virus (HCV).

Examples of retroviral protease inhibitors which can be utilized by the present invention to inhibit pepsin include, but are not limited to, CGP 53437, Amprenavir, Atazanavir, Indinavir, Lopinavir, Fosamprenavir, Nelfinavir, Ritonavir and Saquinavir.

CGP 53437 which demonstrates the highest affinity for pepsin is presently preferred (K_i= 8 nM) [Alteri (1993) Antimicrob. Agents. Chemother. 37:2087-92]. It should be noted that retroviral protease inhibitors which demonstrate low bioavailability and remain in the gastrointestinal lumen are also preferred since use thereof should reduce potential side effects due to the inhibition of other aspartic proteases such as Cathepsin D and β secretase.

Typically, low molecular weight aspartic protease inhibitors irreversibly modify an amino acid residue on the protease active site. One example of a low molecular weight aspartic protease inhibitors which can be utilized by the present invention is pepstatin A. This protease inhibitor is a pentapeptide with a molecular weight of 686 Daltons. It is naturally occurring, secreted by *Streptomyces* bacteria. It is a potent inhibitor of various aspartic proteases including the cathepsin D, the renin, the pepsins, bacterial aspartic proteases and the HIV protease. The prolonged

retention in the stomach at the required site of action, by linking pepstatin to a mucoadhesive polymer, is especially important since it is a small non-specific molecule. Immobilization has the benefit of both slowing clearance from the body and minimizing systemic side effects of the protease inhibitors.

Naturally occurring protease inhibitors have been isolated in a variety of organisms from bacteria to animals and plants. Generally, these behave as tight-binding reversible or pseudo-irreversible inhibitors of proteases preventing substrate access to the active site through steric hindrance. Their sizes typically range from 50 residues (e.g. BPTI: Bovine Pancreatic Trypsin Inhibitor) to 400 residues (e.g. alpha-1PI: alpha-1 Protease Inhibitor) and they are strictly class-specific.

Examples of natural aspartyl protease inhibitors other than pepstatin include, but are not limited to, extracts from *solanum tuberosum* (potato), *Cucurbita maxima* (squash) and *Anchusa strigosa* (Prickly Alkanet) [Strukelj (1990) Nuc. Acid. Res. 18:4605; Farley (2002) J. Mol. Recognit. 15:135-44; Abuereisch (1998) Phytochemistry 48:217-21]. Other potent natural aspartyl protease inhibitors are those originally isolated from the round worms, *Ascaris suum* and *Ascaris lumbricoides*. These natural protease inhibitors include, but are not restricted, Pespin inhibitor III (PI-3) that inactivate pepsins and cathepsin E. These inhibitors are believed to protect the worm from gastric aspartic proteases in the stomach of their host [Abu-Ereish (1974) J. Biol. Chem. 249:1566-71; Kageyama (1998) Eur. J. Bioch. 253:804-9].

Serine protease inhibitors can be used to inhibit the activity of components of the enteropeptidase pathway, as well. These include low or high molecular weight inhibitor groups.

Either synthetic or of bacterial and fungal origin, small serine protease inhibitors irreversibly modify an amino acid residue on the protease active site. Examples of low molecular weight serine protease inhibitors include, but are not limited to, E-64 [Matsushima (1999) Biochem. 125:947-51], antipain, elastatinal, leupeptin, PMSF and its derivative APMSF, benzamidine and its derivative p-aminobenzamidine, chymostatin, TLCK, TPCK, DFP and 3,4-dichloroisocoumarin, all of which are commercially available.

An example of a high molecular weight serine protease inhibitor is the non-peptide based orally active inhibitor of elastase is -(9-(2-piperidinoethoxy)-4-oxo-4H-pyrido 1,2-a pyrimidin-2-yloxymet- hyl)-4-(1-methylethyl)-6-methoxy-1,2-

benzisothiazol-3(2H)-one-1,1-dioxide (SSR69071) [Kapui (2003) Pharmacol Exp Ther 305:451-9].

An example of a peptide based inhibitor of enteropeptidase is Val-(Asp)4-Lys-chloromethyl ketone artificial inhibitor [Antonowicz (1980) Clin. Chim. Acta. 101(1):69-76; Lu (1999) 17:292:361-73].

Natural inhibitors of the enteropeptidase pathway can also be employed by the present invention. Natural serine protease inhibitors are presently the most widely studied of all naturally occurring protease inhibitors. Examples include, α1-protease inhibitor that can be used to inhibit duodenase [Gladysheva (2001) Biochemistry 66(6): 682-7]. Other examples of natural duodenase inhibitors include the inhibitors of the Bowman-Birk family [Gladysheva (2002) Protein Pept. Lett. 9(2): 139-44]. Natural inhibitors of trypsin include the soybean trypsin inhibitor, and extracts of black and white garden beans [Rascon (1985) Comp. Biochem. Physiol. B. 82:375-8]. Natural inhibitors of enteropeptidase include lectins [Rouanet (1983) Experimentia 39:1356-8], kidney bean inhibitors [e.g., EPI purified from red kidney bean, Jacob (1983) Biochem J. 209:91-7; Tajiri (1986) J. Nutr. 116:873-80] and DI which is isolated from bovine duodenum [Mikhailova (1998) Vopr. Med. Khim. 44:338-46].

Another example of an agent capable of downregulating a protein component participating in protein digestion and/or absorption is an antibody or antibody fragment capable of specifically binding the protease, preferably to its active site, thereby preventing its function. For example, amino acids 801-1035 of bovine enteropeptidase, have been determined as its active site [Kitamoto (1994), Proc. Natl. Acad. Sci. USA 91:7588-7592].

The 3 D structure of pepsin renders this protease a good target for antibody manipulation. The antibody can be targeted against its active cleft between its two domains. A flap located over the active site cleft which allows substrate access is another target for antibody recognition [Zlabinger GJ *et al.*, Matrix. 1989 (2):135-9].

Preferably, the antibody specifically binds to at least one epitope of the protein. As used herein, the term "epitope" refers to any antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopes of enteropeptidase catalytic domain preferably include His841, Asp892 and Ser987 [Kitamoto 1994, Proc. Natl. Acad. Sci. USA 91:7588-7592].

Epitopic determinants usually consist of chemically active surface groups of molecules such as amino acids or carbohydrate side chains and usually have specific three-dimensional structural characteristics, as well as specific charge characteristics.

The term "antibody" as used in this invention includes intact molecules as well as functional fragments thereof, such as Fab, F(ab')2, and Fv that are capable of binding to the antigen presented by the macrophages. These functional antibody fragments are defined as follows: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) (Fab')2, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')2 is a dimer of two Fab' fragments held together by two disulfide bridges; (4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (5) Single Chain Antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable peptide linker as a genetically fused single chain molecule.

Methods of producing polyclonal and monoclonal antibodies as well as fragments thereof are well known in the art (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

Antibody fragments according to the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')2. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent

fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R. [Biochem. J. 73: 119-126 (1959)]. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Fv fragments comprise an association of VH and VL chains. This association may be noncovalent, as described in Inbar *et al.*, [Proc. Natl Acad. Sci. USA 69:2659-62 (1972)]. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single peptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described in the litterature [Whitlow and Filpula [(1991), Methods 2: 97-105]; Bird *et al.*, [(1988) Science 242:423-426]; Pack *et al.*, [(1993), BioTechnology 11:1271-77]; and U.S. Pat. No. 4,946,778, which is hereby incorporated by reference.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry [(1991) Human Antibodies and Hybridomas, 2:172-189 and U.S. Pat. No. 6,580,016].

Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab').sub.2 or other antigen-binding subsequences of antibodies) which

contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones *et al.*, Nature, 321:522-525 (1986); Riechmann *et al.*, Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source that is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones *et al.*, Nature, 321:522-525 (1986); Riechmann *et al.*, Nature 332:323-327 (1988); Verhoeyen *et al.*, Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1992); Marks *et al.*, J. Mol. Biol., 222:581 (1991)]. The techniques of Cole

et al., and Boerner *et al.*, are also available for the preparation of human monoclonal antibodies (Cole *et al.*, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner *et al.*, J. Immunol., 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introduction of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,806; 5,545,807;; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks *et al.*, BioTechnology 10,: 779-783 (1992); Lonberg *et al.*, Nature 368: 856-859 (1994); Morrison, Nature 368 812-13 (1994); Fishwild *et al.*, Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14: 826 (1996); and Lonberg and Huszar, Intern. Rev. Immunol. 13, 65-93 (1995).

Alternatively, the agent of this aspect of the present invention may be an oligonucleotide directed against an endogenous nucleic acid sequence expressing the at least one component participating in protein digestion and/or absorption.

In another embodiment, this oligonucleotide (DNA or RNA) is 15 to 30 base pair (bp), preferably 18 to 25 bp long and most preferably 21 bp in length. A oligonucleotide according to the invention is a nucleic acid sequence complementary to the sequences of enteropeptidase or trypsin, and particularly the sequence indicated in Tables 1 and 2. The term "complementary" as defined herein means an oligonucleotide that hybridizes with the sequence to target under its entire length, under stringent conditions (for example, an hybridization carried out between 35 to 65°C using a salt solution which is about 0.9 M). The hybridization may be perfect (100% matching) or imperfect with a mismatch in 1, 2 or 3 nucleotides. An oligonucleotide with some mismatches is considered to be appropriate for the invention if it can direct the degradation of the mRNA, which it is hybridized to.

In a first embodiment, the oligonucleotide is complementary to SEQ ID NO:3 (nucleic acid sequence encoding the human enteropeptidase; SEQ ID NO:4) or a homologue thereof (Table 1). In a second embodiment, the oligonucleotide is complementary to SEQ ID NO:1 (nucleic acid sequence encoding the human trypsin; SEQ ID NO:2) or a homologue thereof (Table 2).

A small interfering RNA (siRNA) molecule is an example of an oligonucleotide agent capable of downregulating a component participating in protein digestion and/or absorption. RNA interference is a two-step process. During the first step, which is termed the initiation step, input dsRNA is digested into 21-23 nucleotides (nt) small interfering RNAs (siRNA), probably by the action of Dicer, a member of the RNase III family of dsRNA-specific ribonucleases, which cleaves dsRNA (introduced directly or *via* an expressing vector, cassette or virus) in an ATP-dependent manner. Successive cleavage events degrade the RNA to 19-21 bp duplexes (siRNA), each strand with 2-nucleotide 3' overhangs [Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002); and Bernstein Nature 409:363-366 (2001)].

In the effector step, the siRNA duplexes bind to a nuclease complex to form the RNA-induced silencing complex (RISC). An ATP-dependent unwinding of the siRNA duplex is required for activation of the RISC. The active RISC then targets the homologous transcript by base pairing interactions and cleaves the mRNA into 12 nucleotide fragments from the 3' terminus of the siRNA [Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002); Hammond *et al.*, (2001) Nat. Rev. Gen. 2:110-119 (2001); and Sharp Genes. Dev. 15:485-90 (2001)]. Although the mechanism of cleavage is still to be elucidated, research indicates that each RISC contains a single siRNA and an RNase [Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002)].

Because of the remarkable potency of RNAi, an amplification step within the RNAi pathway has been suggested. Amplification could occur by copying of the input dsRNAs, which would generate more siRNAs, or by replication of the siRNAs formed. Alternatively or additionally, amplification could be effected by multiple turnover events of the RISC [Hammond *et al.*, Nat. Rev. Gen. 2:110-119 (2001), Sharp Genes. Dev. 15:485-90 (2001); Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002)]. For more information on RNAi see the following reviews Tuschl ChemBiochem. 2:239-245 (2001); Cullen Nat. Immunol. 3:597-599 (2002); and Brantl Biochem. Biophys. Act. 1575:15-25 (2002).

Synthesis of RNAi molecules suitable for use with the present invention can be effected as follows. First, the mRNA sequence target is scanned downstream of the AUG start codon for AA dinucleotide sequences. Occurrence of each AA and the 3'

adjacent 19 nucleotides is recorded as potential siRNA target sites. Preferably, siRNA target sites are selected from the open reading frame, as untranslated regions (UTRs) are richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex [Tuschl ChemBiochem. 2:239-245]. It will be appreciated though, that siRNAs directed at untranslated regions may also be effective, as demonstrated for GAPDH wherein siRNA directed at the 5' UTR mediated about 90 % decrease in cellular GAPDH mRNA and significantly reduced protein level (www.ambion.com/techlib/tn/91/912.html).

Second, potential target sites are compared to an appropriate genomic database (e.g., human, mouse, rat etc.) using any sequence alignment software, such as the BLAST software available from the NCBI server (www.ncbi.nlm.nih.gov/BLAST/). Putative target sites that exhibit significant homology to other coding sequences are filtered out.

Qualifying target sequences are selected as template for siRNA synthesis. Preferred sequences are those including low G/C content as these have proven to be more effective in mediating gene silencing as compared to those with G/C content higher than 55 %. A G/C content comprised between 30 to 50% is preferred. Several target sites are preferably selected along the length of the target gene for evaluation. For better evaluation of the selected siRNAs, a negative control is preferably used in conjunction. Negative control siRNA preferably include the same nucleotide composition as the siRNAs but lack significant homology to the genome. Thus, a scrambled nucleotide sequence of the siRNA is preferably used, provided it does not display any significant homology to any other gene.

Another oligonucleotide agent capable of downregulating a component participating in protein digestion and/or absorption is a DNAzyme molecule capable of specifically cleaving an mRNA transcript or a DNA sequence of the target. DNAzymes are single-stranded polynucleotides which are capable of cleaving both single and double stranded target sequences (Breaker, R.R. and Joyce, G. Chemistry and Biology 1995;2:655; Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 1997;94:4262). A general model (the "10-23" model) for the DNAzyme has been proposed. "10-23" DNAzymes have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides

each. This type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions (Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 199; for rev of DNAzymes see Khachigian, LM [Curr Opin Mol Ther 4:119-21 (2002)].

Examples of construction and amplification of synthetic, engineered DNAzymes recognizing single and double-stranded target cleavage sites have been disclosed in U.S. Pat. No. 6,326,174 to Joyce *et al.* DNAzymes of similar design directed against the human Urokinase receptor were recently observed to inhibit Urokinase receptor expression, and successfully inhibit colon cancer cell metastasis *in vivo* (Itoh *et al.*, 20002, Abstract 409, Ann Meeting Am Soc Gen Ther www.asgt.org). In another application, DNAzymes complementary to bcr-ab1 oncogenes were successful in inhibiting the oncogenes expression in leukemia cells, and lessening relapse rates in autologous bone marrow transplant in cases of Chronic Myelogenous Leukemia (CML) and Acute Lymphocytic Leukemia (ALL).

Downregulation of a component participating in protein digestion and/or absorption can also be effected by using an antisense polynucleotide capable of specifically hybridizing with an mRNA transcript encoding the component participating in protein digestion and/or absorption (e.g., a 21 antisense oligonucleotide directed at the specific enteropeptidase site $R_{96}RRK_{99}$ which is located in the light (catalytic) chain of enteropeptidase).

Design of antisense molecules, which can be used to efficiently down-regulate a component participating in protein digestion and/or absorption, must be effected while considering two aspects important to the antisense approach. The first aspect is delivery of the oligonucleotide into the cytoplasm of the appropriate cells, while the second aspect is design of an oligonucleotide that specifically binds the designated mRNA within cells in a way that inhibits translation thereof.

The prior art teaches of a number of delivery strategies which can be used to efficiently deliver oligonucleotides into a wide variety of cell types [see, for example, Luft J Mol Med 76: 75-6 (1998); Kronenwett *et al.*, Blood 91: 852-62 (1998); Rajur *et al.*, Bioconjug Chem 8: 935-40 (1997); Lavigne *et al.*, Biochem Biophys Res Commun 237: 566-71 (1997) and Aoki *et al.*, (1997) Biochem Biophys Res Commun 231: 540-5 (1997)].

In addition, algorithms for identifying those sequences with the highest predicted binding affinity for their target mRNA based on a thermodynamic cycle that accounts for the energetics of structural alterations in both the target mRNA and the oligonucleotide are also available [see, for example, Walton *et al.*, Biotechnol Bioeng 65: 1-9 (1999)].

Such algorithms have been successfully used to implement an antisense approach in cells. For example, the algorithm developed by Walton *et al.*, enabled scientists to successfully design antisense oligonucleotides for rabbit beta-globin (RBG) and mouse tumor necrosis factor-alpha (TNF alpha) transcripts. The same research group has more recently reported that the antisense activity of rationally selected oligonucleotides against three model target mRNAs (human lactate dehydrogenase A and B and rat gp130) in cell culture as evaluated by a kinetic PCR technique proved to be effective in almost all cases, including tests against three different targets in two cell types with phosphodiester and phosphorothioate oligonucleotide chemistries.

In addition, several approaches for designing and predicting efficiency of specific oligonucleotides using an *in vitro* system were also published (Matveeva *et al.*, Nature Biotechnology 16: 1374 - 1375 (1998)].

Several clinical trials have demonstrated safety, feasibility and activity of antisense oligonucleotides. For example, antisense oligonucleotides suitable for the treatment of cancer have been successfully used [Homlund *et al.*, Curr Opin Mol Ther 1:372-85 (1999)], while treatment of hematological malignancies *via* antisense oligonucleotides targeting c-myb gene, p53 and Bcl-2 had entered clinical trials and had been shown to be tolerated by patients [Gerwitz Curr Opin Mol Ther 1:297-306 (1999)].

More recently, antisense-mediated suppression of human heparanase gene expression has been reported to inhibit pleural dissemination of human cancer cells in a mouse model [Uno *et al.*, Cancer Res 61:7855-60 (2001)].

Thus, the current consensus is that recent developments in the field of antisense technology which, as described above, have led to the generation of highly accurate antisense design algorithms and a wide variety of oligonucleotide delivery systems, enable an ordinarily skilled artisan to design and implement antisense

approaches suitable for downregulating expression of known sequences without having to resort to undue trial and error experimentation.

Another agent capable of downregulating a component participating in protein digestion and/or absorption is a ribozyme molecule capable of specifically cleaving an mRNA transcript encoding a component participating in protein digestion and/or absorption. Ribozymes are being increasingly used for the sequence-specific inhibition of gene expression by the cleavage of mRNAs encoding proteins of interest [Welch *et al.*, Curr Opin Biotechnol. 9:486-96 (1998)]. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications. In the therapeutics area, ribozymes have been exploited to target viral RNAs in infectious diseases, dominant oncogenes in cancers and specific somatic mutations in genetic disorders [Welch *et al.*, Clin Diagn Virol. 10:163-71 (1998)]. Most notably, several ribozyme gene therapy protocols for HIV patients are already in Phase 1 trials. More recently, ribozymes have been used for transgenic animal research, gene target validation and pathway elucidation. Several ribozymes are in various stages of clinical trials. ANGIOZYME was the first chemically synthesized ribozyme to be studied in human clinical trials. ANGIOZYME specifically inhibits formation of the VEGF-r (Vascular Endothelial Growth Factor receptor), a key component in the angiogenesis pathway. Ribozyme Pharmaceuticals, Inc., as well as other firms have demonstrated the importance of anti-angiogenesis therapeutics in animal models. HEPTAZYME, a ribozyme designed to selectively destroy Hepatitis C Virus (HCV) RNA, was found effective in decreasing Hepatitis C viral RNA in cell culture assays (Ribozyme Pharmaceuticals, Incorporated - http://www.rpi.com/index.html).

An additional method of regulating the expression of a component participating in protein digestion and/ or absorption genes in cells is *via* triplex forming oligonuclotides (TFOs). In the last decade, studies have shown that TFOs can be designed which can recognize and bind to polypurine/polypirimidine regions in double-stranded helical DNA in a sequence-specific manner. These recognition rules are outlined by Maher III, L. J., *et al.*, Science (1989) 245:725-730; Moser, H. E., *et al.*, Science (1987)238:645-630; Beal, P. A., *et al.*, Science (1991) 251:1360-1363; Cooney, M., *et al.*, Science(1988)241:456-459; and Hogan, M. E., *et al.*, EP Publication 375408. Modification of the oligonuclotides, such as the introduction of

intercalators and backbone substitutions, and optimization of binding conditions (pH and cation concentration) have aided in overcoming inherent obstacles to TFO activity such as charge repulsion and instability, and it was recently shown that synthetic oligonucleotides can be targeted to specific sequences (for a recent review see Seidman and Glazer (2003) J Clin Invest;112:487-94).

In general, the triplex-forming oligonucleotide has the sequence correspondence:

oligo	3'--A	G	G	T
duplex	5'--A	G	C	T
duplex	3'--T	C	G	A

However, it has been shown that the A-AT and G-GC triplets have the greatest triple helical stability (Reither and Jeltsch (2002), BMC Biochem, , Sept12, Epub). The same authors have demonstrated that TFOs designed according to the A-AT and G-GC rule do not form non-specific triplexes, indicating that the triplex formation is indeed sequence specific.

Thus for any given sequence in the regulatory region a triplex forming sequence may be devised. Triplex-forming oligonucleotides preferably are at least 15, more preferably 25, still more preferably 30 or more nucleotides in length, up to 50 or 100 bp.

Transfection of cells (for example, *via* cationic liposomes) with TFOs, and formation of the triple helical structure with the target DNA induces steric and functional changes, blocking transcription initiation and elongation, allowing the introduction of desired sequence changes in the endogenous DNA and resulting in the specific downregulation of gene expression. Examples of such suppression of gene expression in cells treated with TFOs include knockout of episomal *supFG1* and endogenous *HPRT* genes in mammalian cells (Vasquez *et al.*, Nucl Acids Res. (1999) 27:1176-81, and Puri, *et al.*, J Biol Chem, (2001) 276:28991-98), and the sequence- and target-specific downregulation of expression of the Ets2 transcription factor, important in prostate cancer etiology (Carbone, *et al.*, Nucl Acid Res. (2003) 31:833-43), and the pro-inflammatory ICAM-1 gene (Besch *et al.*, J Biol Chem, (2002) 277:32473-79). In addition, Vuyisich and Beal have recently shown that sequence specific TFOs can bind to dsRNA, inhibiting activity of dsRNA-dependent enzymes

such as RNA-dependent kinases (Vuyisich and Beal, Nuc. Acids Res (2000) ;28:2369-74).

Additionally, TFOs designed according to the abovementioned principles can induce directed mutagenesis capable of effecting DNA repair, thus providing both downregulation and upregulation of expression of endogenous genes [Seidman and Glazer, J Clin Invest (2003) 112:487-94]. Detailed description of the design, synthesis and administration of effective TFOs can be found in U.S. Patent Application Nos. 2003 017068 and 2003 0096980 to Froehler *et al.*, and 2002 0128218 and 2002 0123476 to Emanuele *et al.*, and U.S. Pat. No. 5,721,138 to Lawn.

Additional description of oligonucleotide agents is further provided hereinbelow. It will be appreciated that therapeutic oligonucleotides may further include base and/or backbone modifications, which may increase bioavailability, therapeutic efficacy and reduce cytotoxicity. Such modifications are described in Younes (2002) Current Pharmaceutical Design 8:1451-1466.

For example, the oligonucleotides of the present invention may comprise heterocylic nucleosides consisting of purines and the pyrimidines bases, bonded in a 3' to 5' phosphodiester linkage.

Preferably used oligonucleotides are those modified in either backbone, internucleoside linkages or bases, as is broadly described herein below.

Specific examples of preferred oligonucleotides useful according to this aspect of the present invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. Oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone, as disclosed in U.S. Pat. NOs: 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466, 677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidates and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and

boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms can also be used.

Alternatively, modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts, as disclosed in U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439.

Other oligonucleotides which can be used according to the present invention, are those modified in both sugar and the internucleoside linkage, i.e. the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for complementation with the appropriate polynucleotide target. An example for such an oligonucleotide mimetic includes peptide nucleic acid (PNA). A PNA oligonucleotide refers to an oligonucleotide where the sugar-backbone is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The bases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Other backbone modifications, which can be used in the present invention are disclosed in U.S. Pat. No: 6,303,374.

Oligonucleotides of the present invention may also include base modifications or substitutions. As used herein, "unmodified" or "natural" bases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified bases include but are not limited to other synthetic and natural bases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further bases include those disclosed in U.S. Pat. No: 3,687,808, those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, those disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y. S., Chapter 15, Antisense Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B., ed., CRC Press, 1993. Such bases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 °C. [Sanghvi YS *et al.*, (1993) Antisense Research and Applications, CRC Press, Boca Raton 276-278] and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Examples of oligonucleotide agents which have been used to down-regulate expression of duodenal proteins are described in Ratineau (2004) J. Biol. Chem. 279:24477-84; Khomenko (2003) Biochem. Biophys. Res. Commun. 309:910-6; Morel (1997) Br. J. Pharmacol. 121:451-8.

Alternatively, an agent capable of down-regulating the activity of a component participating in protein digestion and/or absorption can be a non-functional derivative

thereof (i.e. dominant negative). Enteropeptidase forms, which include mutations that render the protein inactive, are known in the art [Holzinger (2002) Am. J. Hum. Genet. 70(1):20-5]. These mutations include, for example, the nonsense mutations S712X, R857X and Q261X, as well as the frameshift mutation FsQ902. At least one of these mutations can be introduced to the subject using the well known "gene knock-in strategy" which will result in the formation of a non-functional protein [see e.g., Matsuda *et al.*, Methods Mol Biol. 2004; 259:379-90]. Alternatively, a non-functional derivative of enteropeptidase can be provided to the subject. Such derivatives may have altered membrane localization, or substrate specificity [Kitamoto (1994) Proc. Natl. Acad. Sci. USA 91:7588-7592].

The amino acid sequence of pepsin together with its 3-D structure makes pepsin a relatively easy target for point mutations and gene knock-in strategy. The enzyme is made up of two domains each of which contributes one aspartic acid residue to the catalytic site. These residues are essential in coordinating a water molecule for nucleophilic attack on the scissile peptide bond. Thus a point mutation in either of these aspartic acid residues would render the protease inactive and could be introduced to the subject using the gene knock-in approach as mentioned herein. An example of a pepsin mutation known in the art includes T77V [Okoniewska *et al.*, Protein Engineering, 1999; 12: 55-61].

Peptides of these non-functional derivatives can be synthesized using solid phase peptide synthesis procedures that are well known in the art and further described by John Morrow Stewart and Janis Dillaha Young, [Solid Phase Peptide Syntheses (2nd Ed., Pierce Chemical Company, 1984)]. Synthetic peptides can be purified by preparative high performance liquid chromatography [Creighton T. (1983) Proteins, structures and molecular principles. WH Freeman and Co. N.Y.] and the composition of which can be confirmed by amino acid sequencing.

In cases where large amounts of the peptide are desired, they can be generated using recombinant techniques such as described by Bitter *et al.*, (1987) Methods in Enzymol. 153:516-544, Studier *et al.*, (1990) Methods in Enzymol. 185:60-89, Brisson *et al.*, (1984) Nature 310:511-514, Takamatsu *et al.*, (1987) EMBO J. 6:307-311, Coruzzi *et al.*, (1984) EMBO J. 3:1671-1680 and Brogli *et al.*, (1984) Science 224:838-843, Gurley *et al.*, (1986) Mol. Cell. Biol. 6:559-565 and Weissbach &

Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463.

Alternatively, these peptides can be manufactured within the target cell by administering a nuclear acid construct of the peptide. It will be appreciated that the nucleic acid construct can be administered to the individual employing any suitable mode of administration, described hereinbelow (i.e. *in vivo* gene therapy). Alternatively, the nucleic acid construct can be introduced into a suitable cell using an appropriate gene delivery vehicle/method (transfection, transduction, etc.) and an appropriate expression system. The modified cells are subsequently expanded in culture and returned to the individual (i.e. *ex vivo* gene therapy). Examples of suitable constructs include, but are not limited to, pcDNA3, pcDNA3.1 (+/-), pGL3, PzeoSV2 (+/-), pDisplay, pEF/myc/cyto, pCMV/myc/cyto each of which is commercially available from Invitrogen Co. (www.invitrogen.com). Examples of retroviral vector and packaging systems are those sold by Clontech, San Diego, Calif., including Retro-X vectors pLNCX and pLXSN, which permit cloning into multiple cloning sites and transcription of the transgene is directed from the CMV promoter. Vectors derived from Mo-MuLV are also included such as pBabe, where the transgene will be transcribed from the 5'LTR promoter.

Currently preferred *in vivo* nucleic acid transfer techniques include infection with viral or transfection with a non-viral constructs. The former includes, but is not limited to the adenovirus, lentivirus, Herpes simplex I virus and adeno-associated virus (AAV) whilst the latter includes, but is not limited to lipid-based systems. Useful lipids for lipid-mediated transfer of the gene are, for example, DOTMA, DOPE, and DC-Chol [Tonkinson *et al.*, Cancer Investigation, 14(1): 54-65 (1996)]. Recently, it has been shown that Chitosan can be used to deliver nucleic acids to the intestine cells (Chen J. (2004) World J Gastroenterol 10(1):112-116). The most preferred constructs for use in gene therapy are viruses, most preferably adenoviruses, AAV, lentiviruses, or retroviruses. A viral construct such as a retroviral construct includes at least one transcriptional promoter/enhancer or locus-defining element(s), or other elements that control gene expression by other means such as alternate splicing, nuclear RNA export, or post-transcriptional modification of messenger. Such vector constructs also include a packaging signal, long terminal repeats (LTRs) or portions thereof, and positive and negative strand primer binding sites appropriate to the virus used, unless

it is already present in the viral construct. In addition, such a construct typically includes a signal sequence for secretion of the peptide from a host cell in which it is placed. Preferably, the signal sequence for this purpose is a mammalian signal sequence or the signal sequence of the peptide variants of the present invention. Optionally, the construct may also include a signal that directs polyadenylation, as well as one or more restriction site and a translation termination sequence. By way of example, such constructs will typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second-strand DNA synthesis, and a 3' LTR or a portion thereof. Other vectors can be used that are non-viral, such as cationic lipids, polylysine, and dendrimers.

As mentioned hereinabove, agents of the present invention can be used for reducing body fat content and as such can be used for treating conditions or disorders associated directly or indirectly with abnormal fat metabolism. Examples include, but are not limited to, overweight, obesity (i.e. at least 20 % over the average weight for the person's age, sex and height), type II diabetes, hyperglycemia, hyperinsulinemia, elevated blood levels of fatty acids or glycerol, syndrome X, diabetic complications, dysmetabolic syndrome and related diseases, sexual dysfunction, hypercholesterolemia, atherosclerosis, hypertension, pancreatitis, hypertriglyceridemia, hyperlipidemia, Alzheimer's disease, osteopenia, stroke, dementia, coronary heart diseases, peripheral vascular diseases, peripheral arterial diseases, vascular syndromes, reducing myocardial revascularization procedures, microvascular diseases (e.g., neuropathy, nephropathy and retinopathy), nephritic syndrome, cholesterol-related disorders (e.g., LDL-pattern B and LDL-pattern L), cerebrovascular diseases, malignant lesions (e.g., ductal carcinoma *in situ*), premalignant lesions, gastrointestinal malignancies (e.g., liposarcoma, epithelial tumors, irritable bowel syndrome, Crohn's disease, gastric ulceritis, gallstones), drug-induced lipodystrophy, inflammatory disorders and climacteric. Agents of the present invention may also be used to treat non-diabetis obesity or non-pancreatitis patients.

It will be appreciated that the agents of the present invention may also be used to modulate body fat content. Thus, for example, agents of the present invention can be used to reduce percent body fat as is often desired by athletes.

As used herein the term "treating" refers to preventing, curing, reversing, attenuating, alleviating, minimizing, suppressing or halting the deleterious effects of

a condition or disorder associated with abnormal fat metabolism symptoms and/or disease state.

The present invention also envisages treating subjects suffering from diseases, in which low-protein diet is typically recommended (in order to reduce symptoms of the disease and make the disease more manageable) with agents of the present invention. Examples of such diseases include, but are not limited to, renal diseases (e.g., chronic renal failure) Parkinson's disease [Riley (1988) Neurology 38:1026-31], Phenylketonuria (PKU), osteoporosis, alkaptonuria (AKU), liver diseases (www.gicare.com/pated/edtgs10.htm), urea cycle disorders and gout (www.cbsnews.com/stories/2004/03/11/health/main605445.shtml).

As used herein in the specification and claims section that follows, the phrase "therapeutically effective amount" refers to an amount which improves at least one of the following criteria: body mass index; % body fat; total body potassium, bioelectrical impedence or under water weighing. As used herein, the body mass index is the ratio between weight (in kilograms) and height squared (in meters square). Total body potassium, which is largely intracellular, is ascertained using a method to detect the natural decay of potassium 40 to potassium 39. This can be used to calculate lean body mass which when subtracted from total body weight will yield body fat mass. The total body potassium method is not widely available for clinical use because it necessitates a spectrometry measurement.

The criteria of bioelectric impedence as used herein is measured using a portable device with paste electrodes which are attached to the right hand and foot. With the patient supine, the total body electrical impedance or resistance is measured. Since water conducts electricity while fat is an insulator, the machine measures body water and calculates body fat. Another method for detecting fat body mass is "Underwater weighing". This method relies on the fact that fat floats in water. Therefore, by comparing body weight on land and underwater, percent body fat can be calculated. Since air also floats, a correction must be made for lung volume, and subjects are encouraged to exhale as they put their heads underwater. This method is especially useful calculating fat body mass in athletes.

The "therapeutically effective amount" will, of course, be dependent on, but not limited to the subject being treated, the severity of the anticipated affliction, the manner of administration, as discussed herein and the judgment of the prescribing

physician. [See e.g. Fingl, *et al.*, (1975) "The Pharmacological Basis of Therapeutics", Ch. 1 p.1].

Determination of a therapeutically effective amount is well within the capability of those skilled in the art. Daily conventional dosages for protease inhibitors may be between 100 to 2000 mg, preferably 500 to 1500 mg, 800 to 1200 mg and most preferably between 800 and 1200 mg, in several timers daily.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from *in vitro* assays. For example, a dose can be formulated in animal models (e.g. obese models such as disclosed by Bayli's J Pharmacol Exp Ther. 2003; and models for atherosclerosis such as described by Brousseau J Lipid Res. (1999) 40(3):365-75 and such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human.

Depending on the severity and responsiveness of the condition to be treated, dosing can be effected over a short period of time (i.e. several days to several weeks) or until cure is effected or diminution of the disease state is achieved.

Agents of the present invention can be provided to the subject *per se*, or as part of a pharmaceutical composition where they are mixed with a pharmaceutically acceptable carrier.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein (i.e. agents) with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the agent preparation, which is accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An

adjuvant is included under these phrases. One of the ingredients included in the pharmaceutically acceptable carrier can be for example polyethylene glycol (PEG), a biocompatible polymer with a wide range of solubility in both organic and aqueous media (Mutter *et al.*, (1979).

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. The preferred route of administration is presently oral.

Carrier systems such as micro-spheres and nanoparticles that can improve the bioavailability of the agents may be preferably used in conjunction with the present invention [see Pappas (2004) Expert Opin. Biol. Ther. 4:881-7; Cefalu (2004) Drugs 64:1149-61; and Gowthamarajan and Kulkarni (2003) Resonance 38-46]. Additionally, microemulsion formulations offer improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity [Constantinides *et al.*, Pharmaceutical Research, 1994, 11:1385; Ho *et al.*, J. Pharm. Sci., 1996, 85:138-143].

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically

acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize, wheat, rice, or potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions, which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches,

lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The preparations described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The preparation of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Compositions including the preparation of the present invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

As mentioned hereinabove, agents of the present invention may also be used for reducing body fat content in animals such as domestic animals. In this case agents of the present invention may be administered, dispersed in, or mixed with, animal feedstuff, drinking water and other liquids normally consumed by the animals, or in compositions containing the agents of the present invention dispersed in or mixed with any other suitable inert physiologically acceptable carrier or diluent which is preferably orally administrable (as defined hereinabove). Such compositions may be administered in the form of powders, pellets, solutions, suspensions and emulsions, to the animals to supply the desired dosage of the agents of the present invention or used as concentrates or supplements to be diluted with additional carrier, feed-stuff, drinking water or other liquids normally consumed by the animals, before administration. Suitable inert physiologically acceptable carriers or diluents include wheat flour or meal, maize gluten, lactose, glucose, sucrose, talc, kaolin, calcium phosphate, potassium sulphate and diatomaceous earths such as keiselguhr. Concentrates or supplements intended for incorporation into drinking water or other liquids normally consumed by the animals to give solutions, emulsions or stable

suspensions, may also include the active agent in association with a surface-active wetting, dispersing or emulsifying agent such as Teepol, polyoxyethylene (20) sorbitan mono-oleate or the condensation product of β-naphthalenesulphonic acid with formaldehyde, with or without a physiologically innocuous, preferably water-soluble, carrier or diluent, for example, sucrose, glucose or an inorganic salt such as potassium sulphate, or concentrates or supplements in the form of stable dispersions or solutions obtained by mixing the aforesaid concentrates or supplements with water or some other suitable physiologically innocuous inert liquid carrier or diluent, or mixtures thereof (see U.S. Pat. No. 4,005,217).

Each of the agents described hereinabove is administered to the treated subject for a time period sufficient to prevent degradation of essential proteins which may be life threatening (see Guyton and Hall "The Textbook of Medical Physiology" 10th Ed. Harcourt International Edition).

It will be appreciated that the agents of the present invention may be administered in combination with other drugs to achieve enhanced effects (e.g., see Background section and WO 2004/037159 to Harosh).

It will be further appreciated that the agents of the present invention may also be provided as food additives.

The phrase "food additive" [defined by the FDA in 21 C.F.R. 170.3(e)(1)] includes any liquid or solid material intended to be added to a food product. This material can, for example, include an agent having a distinct taste and/or flavor or a physiological effect (e.g., vitamins).

The food additive composition of the present invention can be added to a variety of food products.

As used herein, the phrase "food product" describes a material consisting essentially of protein, carbohydrate and/or fat, which is used in the body of an organism to sustain growth, repair and vital processes and to furnish energy. Food products may also contain supplementary substances such as minerals, vitamins and condiments. See Merriani-Webster's Collegiate Dictionary, 10th Edition, 1993. The phrase "food product" as used herein further includes a beverage adapted for human or animal consumption.

A food product containing the food additive of the present invention can also include additional additives such as, for example, antioxidants, sweeteners, flavorings,

colors, preservatives, nutritive additives such as vitamins and minerals, amino acids (i.e. essential amino acids), emulsifiers, pH control agents such as acidulants, hydrocolloids, antifoams and release agents, flour improving or strengthening agents, raising or leavening agents, gases and chelating agents, the utility and effects of which are well-known in the art.

The present invention also concerns a composition comprising an agent capable of down-regulating activity and/or expression of at least one component participating in protein digestion and/or absorption as defined above, for use in the reduction of percent body fat or for treating conditions or disorders associated directly or indirectly with abnormal fat metabolism.

Moreover, the use of an agent capable of down-regulating activity and/or expression of at least one component participating in protein digestion and/or absorption as defined above, in the manufacture of a composition or a drug for the treatment of conditions or disorders associated directly or indirectly with abnormal fat metabolism also is part of the invention.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

EXAMPLES

Example 1: *in vitro* testing: the trypsinogen activation assay

Material: The following component, used in the present trypsinogen activation assay may be purchased as follows:

Table 3

Component	**Purchaser; catalogue number**
Recombinant human enteropeptidase	R&D Systems; 1585-SE.
N-CBZ-Gly-Pro-Arg-pnitroanilide	SIGMA; C2276
Trypsinogen	SIGMA; T-1143
AC-Leu-Val-Lys-Aldhehyde [2]	Bachem; N-1380 (4020266)
BOC-Ala-Glu-Val-Asp-Aldehyde [1]	Bachem; N-1755 (4029153)
H-D-Tyr-Pro-Arg-chloromethylketone trifluroroacetate salt [2]	Bachem; N-1225 (40173722)
Z-Asp-Glu-Val-Asp-chloromethylketone [2]	Bachem; N-1580 (4027524)
1,5-Dansyl-Glu-Gly-Arg- chloromethylketone dihydrochloride [1]	Calbiochem; 251700

[1] negative control; [2] candidate molecule

Method

The trypsinogen activation assay is shown in Figure 5. In the first step, the enteropeptidase cleaves the trypsinogen in its active form, trypsin. Trypsin, in the second step, cleaves the N-CBZ-Gly-Pro-Arg-pnitroanilide (pNA) into N-CBZ-Gly-Pro-Arg and pnitroanilide (pNA). The amount of pNA can be measured at 405 nm, and reflects the amount of trypsin cleaved and thus the inhibitory activity of the molecules tested on the enteropeptidase.

In the first step, the following mix was prepared (50 µl final)

- recombinant human enteropeptidase: 1.5 nM final
- sodium citrate: 50 nM final
- candidate molecule or control: 1 µl
- trypsinogen: 2.5 µM final

The mix was incubated at room temperature during 10 minutes and the reaction was stopped with 5 µl of HCl 0.4 M.

In the second step, the previous mix was then incubated with a 50 µl mix comprising 1mM of N-CBZ-Gly-Pro-Arg-pNA, Tris Hcl pH 8.4 20mM final and NaCl 150 mM final, at room temperature for 10 minutes. The absorbance of the resulting mix was read at 405nm.

Results are expressed as the percentage of inhibition, which is the absorbance at 405 nm of the reaction in the presence of different concentrations of inhibitor as compared to the value obtained in the absence of inhibitor.

Results

Control molecules (BOC-Ala-Glu-Val-Asp-Aldehyde and Z-Asp-Glu-Val-Asp-chloromethylketone) were tested at high concentration (10 and 50 µm respectively). As expected, no inhibition was observed, since these two molecules contain an aspartate residue at position P1 which is not expected to be recognised by enteropeptidase.

In contrast the three candidate molecules, tested for their suspected inhibition activity, show a 50% inhibition (as compared to values in absence of inhibitors) at very low concentrations. The IC50 measurement was performed using a Prism graphic application. Graphic representation and IC50 value for these candidate molecules are shown in Figure 6A (AC-Leu-Val-Lys-Aldhehyde), Figure 6B (H-D-Tyr-Pro-Arg-chloromethylketone trifluroroacetate salt) and Figure 6C (1,5-Dansyl-Glu-Gly-Arg-chloromethylketone dihydrochloride).

The IC50 was about 3 µM for AC-Leu-Val-Lys-Aldhehyde, and about 35 and 24.7 nM for H-D-Tyr-Pro-Arg-chloromethylketone trifluroroacetate salt and 1,5-Dansyl-Glu-Gly-Arg-chloromethylketone dihydrochloride respectively.

Additional experiments have shown that H-D-Tyr-Pro-Arg-chloromethylketone trifluroroacetate salt and Z-Asp-Glu-Val-Asp-chloromethylketone molecules, when tested for enteropeptidase only, give a higher IC50 than the ones reported in Figure 6 (data not shown).

Consequently, these observations show that candidate molecules able to compete with both trypsinogen and substrate of tryspin give a cumulative effect on

inhibition of tryspin activity; first directly, by inhibiting the activity of tryspin, and also indirectly by inhibiting the activity of enteropeptidase.

Due to their low IC50 value, these molecules are excellent candidates for the enteropeptidase activity inhibition.

Example 2: *in vivo* testing in rats

To test the effects of molecules on the reduction of body fat, 30 male, genetically obese Zucker rats (Charles River Laboratories ; strain: Crl: ZUC (Orl)-Lepr^{fa}) having an age of 16 weeks at the beginning of this study are utilized. Zucker rats have an autosomal recessive mutation that results in obesity. 30 Zucker rats are divided into 6 groups (5 rats in each group) of which:

- 1 group is used as a control and received water only;
- 1 group is given a mix of 5 particular antisense oligonucleotides (Table 5 below),
- 2 groups receive H-D-Tyr-Pro-Arg-chloromethylketone trifluroroacetate salt (two concentrations),
- 2 groups receive Z-Asp-Glu-Val-Asp-chloromethylketone (two concentrations).

The 5 groups (2 to 6) all receive the candidate molecules in the same vehicle (water). The treatment is administered orally (gavage) one time per day, 15 to 30 minutes before food intake during 28 consecutive days, under conditions indicated in Table 4.

Table 4

Group	Test item	Test item mg / kg of rat	Concentration of test item
1	Vehicle (water)	/	/
2	Mix of oligonucleotides	0.04[(1)]	0.004
3	H-D-Tyr-Pro-Arg-chloromethylketone trifluroroacetate salt	1	0.1
4	H-D-Tyr-Pro-Arg-chloromethylketone trifluroroacetate salt	4	0.4
5	Z-Asp-Glu-Val-Asp-chloromethylketone	1	0.1
6	Z-Asp-Glu-Val-Asp-chloromethylketone	4	0.4

[(1)] 0.04 mg of each oligonucleotide / kg of rat

The period of acclimation lasts for the first five days wherein food is given *ad libitum*. After the initial acclimation period of five days, the next fourteen days, the rats are conditioned by restricting their food intake by a period of three hours per day. All rats are given a hyper-protein food of 25 to 30%. . The rats are observed 1 time per day. Their weight is monitored every 3 days during the 14 day period.

Testing Specific Oligonucleotides in the Zucker Rat

The sequences of the oligonucleotides chosen for this study are sequences that are complementary to the enteropeptidase nucleic acid of the rat and should recognize, within the cell, the mRNA of rat enteropeptidase. This heterocomplex of RNA:oligonucleotide induces the activation of RNase H which degrades the RNA strand. The oligonucleotides have about 20 bases and are protected from degradation by nucleases due to the modification of type 2'-O methyl in position 5' (m) of the three last nucleotides.

5 oligonucleotides are chosen from the sequence of enteropeptidase and the name is the first position of the sequence on the enteropeptidase. These sequences are set forth in Table 5 below:

Table 5

Number	Name of oligonucleotide	Sequence (from 5 to 3')	SEQ ID
1	ODN2053	CCTGCCTGGGTGTCACTTCmCmAmC	5
2	ODN2154	GCAGCAGACACCAGCCAGTCmAmAmU	6
3	ODN1160	GTAGGATGCTCTGGTGGAmGmGmG	7
4	ODN2689	CCCAGGGTGATTAGGCAGTGmCmAmC	8
5	ODN1527	CCTGGCAGGGCTGTGGAATmCmCmC	9

m: 2'O-methyl modification in position 5'

40 µg/kg of each of the above oligonucleotides are given to each of the Zucker rats orally for 28 days.

Testing molecules

H-D-Tyr-Pro-Arg-chloromethylketone trifluroroacetate salt and Z-Asp-Glu-Val-Asp-chloromethylketone are ordered from Bachem, and are available under reference N-1225 (40173722) and N-1580 (4027524). H-D-Tyr-Pro-Arg-chloromethylketone trifluroroacetate salt has a molecular formula $C_{21}H_{31}ClN_6O_4$, a relative molecular weight of 466.97 and a degree of purity of 91%. Z-Asp-Glu-Val-Asp-chloromethylketone has a molecular formula of $C_{27}H_{35}N4O_{12}Cl$, a relative molecular weight of 643.10 and a degree of purity more than 95%.

H-D-Tyr-Pro-Arg-chloromethylketone trifluroroacetate salt is used *in vivo* as a candidate molecule, since it gives excellent IC50 in *in vitro* experiment.

Z-Asp-Glu-Val-Asp-chloromethylketone, shown to not inhibit the enteropeptisae and trypsin, is used as a side effect control. Indeed, the chloromethylketone group may irritate the esophagus, and thus reduce the amount of candidate molecule ingerate due to lesion. This molecule may therefore, in the absence of inhibition of enteropeptisae and trypsin, enable the distinction between a loss of weight due to the candidate molecule (in the case of the H-D-Tyr-Pro-Arg-chloromethylketone trifluroroacetate) and a loss of weight due to esophagus injury.

At day 14 and at the end of the 28 days each of the rats are bled. Total protein, total cholesterol, HDL, LDL, glucose and triglycerides are measured using kits from HORIBA.ABX (Montpelier, France), according to the manufacturer's instructions.

Example 3

The same conditions as the one described in example 2 are used in this example. Male obese Zucker rats are administrated with one or combination (2, 3, 4 or 5) oligonucleotides disclosed in Table 5.

Each of the rats, that are administered oligonucleotide numbers 1 to 5 or combination thereof, experiences a decrease in the levels of total protein, total cholesterol, LDL, glucose and triglycerides as compared to the control group. An increase in HDL is observed in the rats that are administered oligonucleotide numbers 1 to 5 or combination thereof, as compared to the control group.

The final weight of the rats is also undertaken. The rats administered the oligonucleotides numbers 1 to 5 or combination thereof experience a reduction in weight loss as compared to that of the control.

Example 4: Treating Obesity

A group of obese men and women are used in this example. Obesity is determined by their body mass index (BMI) kg/m^2. A value of over 30 kg/m^2 or greater is considered to be obese. 10 females having an average age of 30 years and 10 males having an average age of 40 years are used in this example. All of the people have a body mass index of over 30 kg/m^2, and more particularly ranging from 30 to 35 kg/m^2, which is indicative of obesity.

The study group is advised to follow their normal routine concerning their eating habits and exercise patterns, which is recorded 1 month prior to this study and throughout this study.

5 females and 5 males are given a treatment of ritonavir at 600 mg taken twice a day. The other group of 5 females and 5 males is given a placebo twice a day. The treatment continued for 2 months. At the end of two months another body mass index is taken of the control group and the treated group. The body mass index of the treated group decreased by a factor of 3 kg/m^2 to 5 kg/m^2 at the end of the two month period; i.e., an average weight loss between 20 and 30 pounds, while the mass body index of the control group remained unchanged.

Example 5: Treating Obesity

The same study is done as in Example 4, however different protease inhibitors are used in this study such as amprenavir, atazanavir, indinavir, lopinavir, fosamprenavir, nelfinavir or saquinavir. A larger group study is undertaken using 20 females and 20 males having an average age of 38 years and having a body mass index ranging from 30 to 40 kg/m^2. 7 groups of 4 (2 females & 2 males) are given one of the following doses of protease inhibitors:

Group 1: Amprenavir: 1,200 mg twice a day

Group 2: Atazanavir: 400 mg once a day

Group 3: Indinavir: 800 mg every 8 hours

Group 4: Lopinavir: 399 mg twice a day

Group 5: Fosamprenavir: 1,400 mg two times a day

Group 6: Nelfinavir: 750 mg three times a day

Group 7: Saquinavir: 1,000 mg twice a day

The remaining group of 6 males and 6 females are given a placebo. The treatment continued for 2 months. At the end of two months another body mass index is taken of the control group and the treated group. The body mass index of the treated group decreases by a factor of 3 kg/m^2 to 5 kg/m^2 at the end of the two month period; i.e., an average weight loss between 20 and 30 pounds, while the mass body index of the control group remained unchanged.

Example 6: Treating Type II Diabetes

Type II diabetes is a disease in which the amount of insulin produced by the pancreas is inadequate to meet the body's needs and thus glucose, which is metabolized by insulin is not taken up normally from the blood into the body tissues. Therefore glucose in the blood rises. Type II diabetes is detected by a fasting glucose level of greater than 126 mg/dL measured on two occasions or one blood glucose level of greater than 200 mg/dL on one occasion or two random blood glucose levels of more than 200 mg/dL.Also a glucose tolerance test having a glucose level of more than 200 mg/dL 2 hours after drinking 75 grams of glucose also qualifies an individual as having Type II diabetes.

Two groups of 10 people are used in this study. The first given a treatment of ritonavir at 600 mg taken twice a day, while the other 10 people were given a placebo. The treatment continued for 2 months.

The study group is advised to follow their normal routine concerning their eating habits and exercise patterns, which is recorded 1 month prior to this study and throughout this study.

At the end of two months blood glucose levels and a glucose tolerance test are tested with all of the people in the study. The people given ritonavir have significantly reduced levels of blood glucose than those in the control group.

Example 7: Treating Type II Diabetes

The same study in Example 6 is conducted with a larger group of people having Type II diabetes. Each of the treated groups 1 to 7 is given as amprenavir, atazanavir, indinavir, lopinavir, fosamprenavir, nelfinavir or saquinavir in the same amounts as set forth in Example 3. The control group is given a placebo. At the end of two months another fasting (9-12 hours) lipid profile is taken. The people given amprenavir, atazanavir, indinavir, lopinavir, fosamprenavir, nelfinavir or saquinavir have significantly reduced levels of blood glucose than those in the control group.

Example 8: Treating Hyperlipidemia

Hyperlipidemia is an elevation of lipids in the bloodstream. These lipids include, for example, cholesterol and triglycerides. General hyperlipidemia is determined by the results of a lipid profile. The lipid profile includes LDL, HDL, triglycerides and total cholesterol measurements. A group of persons having hyperlipidemia with a total cholesterol level greater than 240 mg/dl, an HDL (high density lipid) of below 40 mg/ml, a triglyceride level of greater than 200 mg/dl and an LDL (low density lipid) level of over 160 mg/ml, after a 9 to 12 hours of fasting, are chosen for this study.

10 people are given a treatment of ritonavir at 600 mg taken twice a day. The other 10 people are given a placebo. The treatment continued for 2 months.

The study group is advised to follow their normal routine concerning their eating habits and exercise patterns, which is recorded 1 month prior to this study and throughout this study.

At the end of two months another fasting (9-12 hours) lipid profile is taken. The people given ritonavir have significantly reduced levels of total cholesterol, triglycerides and LDL and higher levels of HDL than those in the control group.

Example 9: Treating Hyperlipidemia

The same study in Example 8 is conducted with a larger group of people having hyperlipidemia. Each of the treated groups 1 to 7 is given as amprenavir, atazanavir, indinavir, lopinavir, fosamprenavir, nelfinavir or saquinavir in the same

amounts as set forth in Example 3. The control group is given a placebo. At the end of two months another fasting (9-12 hours) lipid profile is taken. The people given amprenavir, atazanavir, indinavir, lopinavir, fosamprenavir, nelfinavir or saquinavir have significantly reduced levels of cholesterol, triglycerides and LDL and higher levels of HDL than those in the control group.

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CLAIMS

1. A method of reducing body fat content of a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an agent capable of down-regulating activity and/or expression of at least one component participating in protein digestion and/or absorption.

2. The method of claim 1, wherein said component participating in protein digestion and/or absorption is a protease.

3. The method of claim 2, wherein said protease is at least one component of an enteropeptidase pathway.

4. The method of claim 3, wherein said at least one component of an enteropeptidase pathway is a serine-protease.

5. The method of claim 3, wherein said at least one component of an enteropeptidase pathway is an activator of enteropeptidase.

6. The method of claim 4, wherein said at least one component of an enteropeptidase pathway is enteropeptidase.

7. The method of claim 3, wherein said at least one component of an enteropeptidase pathway is a downstream effector of enteropeptidase.

8. The method of claim 7, wherein said downstream effector of enteropeptidase is trypsin.

9. The method of claim 2, wherein said protease is an aspartate-protease.

10. The method of claim 9, wherein said protease is a pepsin.

11. The method of claim 10, wherein said pepsin is selected from the group consisting of Pepsin A, Pepsin B and Gastricin.

12. The method of claim 1, wherein down-regulating activity and/or expression of at least one component participating in protein digestion and/or absorption is effected by an agent selected from the group consisting of:
    (i) an oligonucleotide directed to an endogenous nucleic acid sequence expressing said at least one component participating in said protein digestion and/or absorption;
    (ii) a protease inhibitor directed to said at least one component participating in protein digestion and/or absorption

13. The method of claim 12, wherein said protease inhibitor is an aspartic protease inhibitor.

14. The method of claim 13, wherein said aspartic protease inhibitor is a peptidomimetic aspartic protease inhibitor.

15. The method of claim 13, wherein said aspartic protease inhibitor is a low molecular weight aspartic protease inhibitor.

16. The method of claim 15, wherein said low molecular weight aspartic protease inhibitor is pepstatin.

17. The method of claim 13, wherein said aspartic protease inhibitor is extracted from a plant.

18. The method of claim 17, wherein said plant is selected from the group consisting of *Solanum tuberosum* (potato), *Cucurbita maxima* (squash) and *Anchusa strigosa* (Prickly Alkanet).

19. The method of claim 13, wherein said aspartic protease inhibitor is extracted from a parasite.

20. The method of claim 19, wherein said parasite is selected from the group *Ascaris suum* and *Ascaris lombricoides*.

21. The method of claim 19, wherein said aspartic protease inhibitor is PI-3.

22. The method of claim 12, wherein said protease inhibitor is a serine protease inhibitor.

23. The method of claim 22, wherein said protease inhibitor is an inhibitor of enteropeptidase.

24. The method of claim 22, wherein said protease inhibitor is an inhibitor of trypsin.

25. The method of claim 12 wherein said oligonucleotide is DNA or RNA.

26. The method of claim 25 wherein said oligonucleotide is complementary to SEQ ID NO:1 or homologues thereof.

27. The method of claim 25 wherein said oligonucleotide is complementary to SEQ ID NO:2 or homologues thereof.

28. The method of claim 22, wherein said serine protease inhibitor is a low molecular weight serine protease inhibitor.

29. The method of claim 22, wherein said serine protease inhibitor is a peptidomimetic serine protease inhibitor.

30. The method of claim 22 wherein said serine protease inhibitor is an inhibitor of both enteropeptidase and trypsin.

31. The method of claim 1, wherein said agent is linked to a mucoadhesive agent.

32. The method of claim 31, wherein said mucoadhesive agent is a mucoadhesive polymer.

33. The method of claim 32, wherein said mucoadhesive polymer is selected from the group consisting of chitosan, polyacrylic acid, hydroxyprpyl methylcellulose and hyaluronic acid.

34. The method of claim 1, wherein said subject in need thereof is afflicted with a condition or disorder selected from the group consisting of excessive weight, obesity, type II diabetes, hypercholesterolemia, atherosclerosis, hypertension, pancreatitis, hypertriglyceridemia and hyperlipidemia, or is a non-diabetic or non-pancreatitis patient.

35. The method of claim 1, wherein said administering to the subject is effected by oral administration.

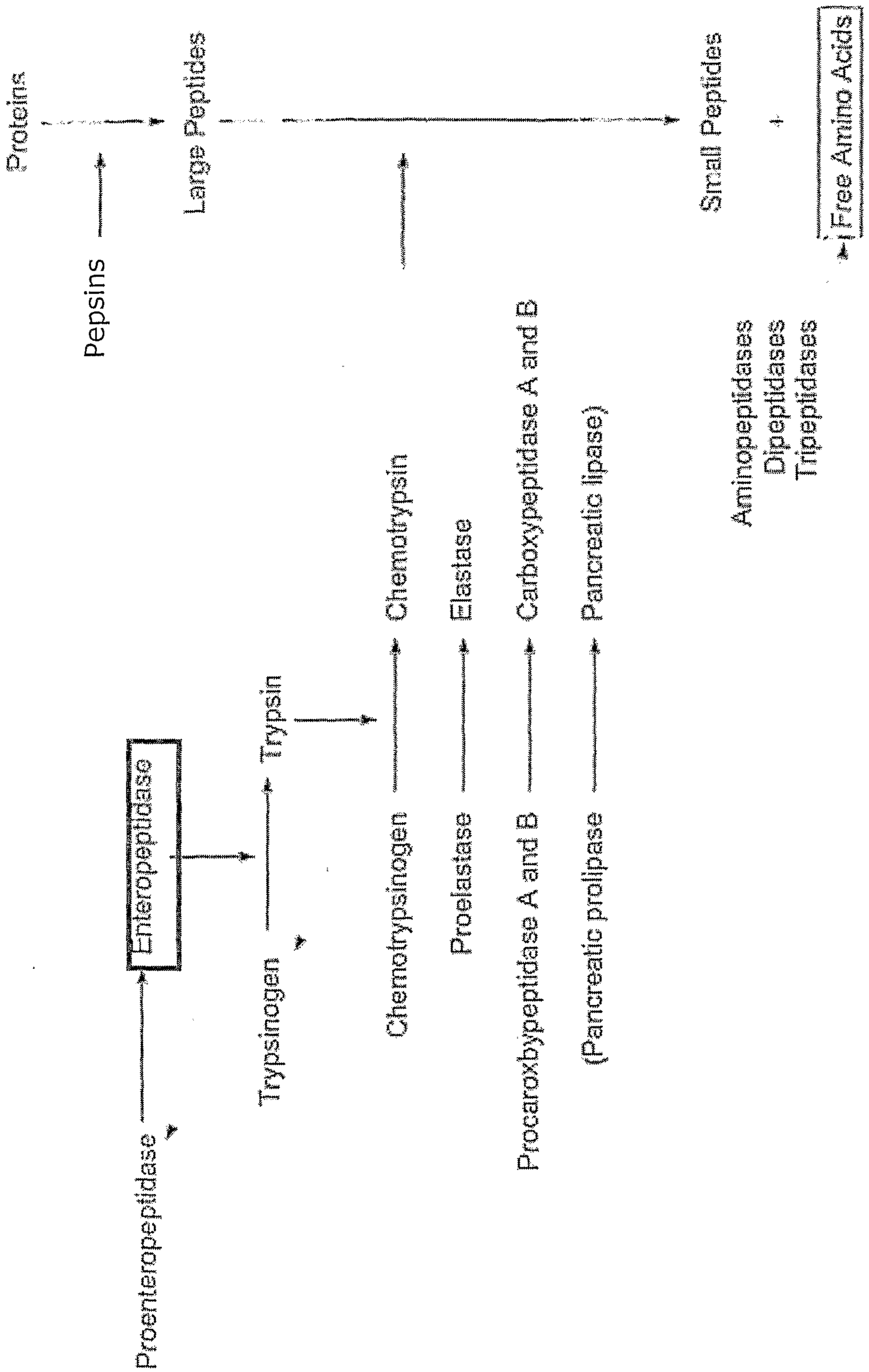
Proteins
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Fig. 1

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       Met Asn Pro Leu Leu Ile Leu Thr Phe Val Ala Ala Ala Leu
       1               5                   10

gct gcc ccc ttt gat gat gat gac aag atc gtt ggg ggc tac aac tgt          96
Ala Ala Pro Phe Asp Asp Asp Asp Lys Ile Val Gly Gly Tyr Asn Cys
15                  20                  25                  30

gag gag aat tct gtc ccc tac cag gtg tcc ctg aat tct ggc tac cac         144
Glu Glu Asn Ser Val Pro Tyr Gln Val Ser Leu Asn Ser Gly Tyr His
                35                  40                  45

ttc tgt ggt ggc tcc ctc atc aac gaa cag tgg gtg gta tca gca ggc         192
Phe Cys Gly Gly Ser Leu Ile Asn Glu Gln Trp Val Val Ser Ala Gly
            50                  55                  60

cac tgc tac aag tcc cgc atc cag gtg aga ctg gga gag cac aac atc         240
His Cys Tyr Lys Ser Arg Ile Gln Val Arg Leu Gly Glu His Asn Ile
        65                  70                  75

gaa gtc ctg gag ggg aat gag cag ttc atc aat gca gcc aag atc atc         288
Glu Val Leu Glu Gly Asn Glu Gln Phe Ile Asn Ala Ala Lys Ile Ile
    80                  85                  90

cgc cac ccc caa tac gac agg aag act ctg aac aat gac atc atg tta         336
Arg His Pro Gln Tyr Asp Arg Lys Thr Leu Asn Asn Asp Ile Met Leu
95                  100                 105                 110

atc aag ctc tcc tca cgt gca gta atc aac gcc cgc gtg tcc acc atc         384
Ile Lys Leu Ser Ser Arg Ala Val Ile Asn Ala Arg Val Ser Thr Ile
                115                 120                 125

tct ctg ccc acc gcc cct cca gcc act ggc acg aag tgc ctc atc tct         432
Ser Leu Pro Thr Ala Pro Pro Ala Thr Gly Thr Lys Cys Leu Ile Ser
            130                 135                 140

ggc tgg ggc aac act gcg agc tct ggc gcc gac tac cca gac gag ctg         480
Gly Trp Gly Asn Thr Ala Ser Ser Gly Ala Asp Tyr Pro Asp Glu Leu
        145                 150                 155

cag tgc ctg gac gct cct gtg ctg agc cag gct aag tgt gaa gcc tcc         528
Gln Cys Leu Asp Ala Pro Val Leu Ser Gln Ala Lys Cys Glu Ala Ser
    160                 165                 170

tac cct gga aag att acc agc aac atg ttc tgt gtg ggc ttc ctt gag         576
Tyr Pro Gly Lys Ile Thr Ser Asn Met Phe Cys Val Gly Phe Leu Glu
175                 180                 185                 190

gga ggc aag gat tca tgt cag ggt gat tct ggt ggc cct gtg gtc tgc         624
Gly Gly Lys Asp Ser Cys Gln Gly Asp Ser Gly Gly Pro Val Val Cys
                195                 200                 205

aat gga cag ctc caa gga gtt gtc tcc tgg ggt gat ggc tgt gcc cag         672
Asn Gly Gln Leu Gln Gly Val Val Ser Trp Gly Asp Gly Cys Ala Gln
            210                 215                 220
```

Fig. 2A

```
aag aac aag cct gga gtc tac acc aag gtc tac aac tat gtg aaa tgg      720
Lys Asn Lys Pro Gly Val Tyr Thr Lys Val Tyr Asn Tyr Val Lys Trp
        225                 230                 235

att aag aac acc ata gct gcc aat agc taa agccccagt atctcttcag        770
Ile Lys Asn Thr Ile Ala Ala Asn Ser
    240                 245

tctctatacc aataaagtga ccctgttctc                                    800
```

Fig. 2B

```
accagacagt tcttaaatta gcaagccttc aaaaccaaaa atg ggg tcg aaa aga        55
                                               Met Gly Ser Lys Arg
                                               1               5

ggc ata tct tct agg cat cat tct ctc agc tcc tat gaa atc atg ttt       103
Gly Ile Ser Ser Arg His His Ser Leu Ser Ser Tyr Glu Ile Met Phe
                10                  15                  20

gca gct ctc ttt gcc ata ttg gta gtg ctc tgt gct gga tta att gca       151
Ala Ala Leu Phe Ala Ile Leu Val Val Leu Cys Ala Gly Leu Ile Ala
            25                  30                  35

gta tcc tgc ctg aca atc aag gaa tcc caa cga ggt gca gca ctt gga       199
Val Ser Cys Leu Thr Ile Lys Glu Ser Gln Arg Gly Ala Ala Leu Gly
        40                  45                  50

cag agt cat gaa gcc aga gcg aca ttt aaa ata aca tcc gga gtt aca       247
Gln Ser His Glu Ala Arg Ala Thr Phe Lys Ile Thr Ser Gly Val Thr
    55                  60                  65

tat aat cct aat ttg caa gac aaa ctc tca gtg gat ttc aaa gtt ctt       295
Tyr Asn Pro Asn Leu Gln Asp Lys Leu Ser Val Asp Phe Lys Val Leu
70                  75                  80                  85

gct ttt gac ctt cag caa atg ata gat gag atc ttt cta tca agc aat       343
Ala Phe Asp Leu Gln Gln Met Ile Asp Glu Ile Phe Leu Ser Ser Asn
                90                  95                  100

ctg aag aat gaa tat aag aac tca aga gtt tta caa ttt gaa aat ggc       391
Leu Lys Asn Glu Tyr Lys Asn Ser Arg Val Leu Gln Phe Glu Asn Gly
            105                 110                 115

agc att ata gtc gta ttt gac ctt ttc ttt gcc cag tgg gtg tca gat       439
Ser Ile Ile Val Val Phe Asp Leu Phe Phe Ala Gln Trp Val Ser Asp
        120                 125                 130

caa aat gta aaa gaa gaa ctg att caa ggc ctt gaa gca aat aaa tcc       487
Gln Asn Val Lys Glu Glu Leu Ile Gln Gly Leu Glu Ala Asn Lys Ser
    135                 140                 145

agc caa ctg gtc act ttc cat att gat ttg aac agc gtt gat atc cta       535
Ser Gln Leu Val Thr Phe His Ile Asp Leu Asn Ser Val Asp Ile Leu
150                 155                 160                 165

gac aag cta aca acc acc agt cat ctg gca act cca gga aat gtc tca       583
Asp Lys Leu Thr Thr Thr Ser His Leu Ala Thr Pro Gly Asn Val Ser
                170                 175                 180

ata gag tgc ctg cct ggt tca agt cct tgt act gat gct cta acg tgt       631
Ile Glu Cys Leu Pro Gly Ser Ser Pro Cys Thr Asp Ala Leu Thr Cys
            185                 190                 195
```

Fig. 3A

```
ata aaa gct gat tta ttt tgt gat gga gaa gta aac tgt cca gat ggt      679
Ile Lys Ala Asp Leu Phe Cys Asp Gly Glu Val Asn Cys Pro Asp Gly
        200                 205                 210

tct gac gaa gac aat aaa atg tgt gcc aca gtt tgt gat gga aga ttt      727
Ser Asp Glu Asp Asn Lys Met Cys Ala Thr Val Cys Asp Gly Arg Phe
    215                 220                 225

ttg tta act gga tca tct ggg tct ttc cag gct act cat tat cca aaa      775
Leu Leu Thr Gly Ser Ser Gly Ser Phe Gln Ala Thr His Tyr Pro Lys
230                 235                 240                 245

cct tct gaa aca agt gtt gtc tgc cag tgg atc ata cgt gta aac caa      823
Pro Ser Glu Thr Ser Val Val Cys Gln Trp Ile Ile Arg Val Asn Gln
                250                 255                 260

gga ctt tcc att aaa ctg agc ttc gat gat ttt aat aca tat tat aca      871
Gly Leu Ser Ile Lys Leu Ser Phe Asp Asp Phe Asn Thr Tyr Tyr Thr
            265                 270                 275

gat ata tta gat att tat gaa ggt gta gga tca agc aag att tta aga      919
Asp Ile Leu Asp Ile Tyr Glu Gly Val Gly Ser Ser Lys Ile Leu Arg
        280                 285                 290

gct tct att tgg gaa act aat cct ggc aca ata aga att ttt tcc aac      967
Ala Ser Ile Trp Glu Thr Asn Pro Gly Thr Ile Arg Ile Phe Ser Asn
    295                 300                 305

caa gtt act gcc acc ttt ctt ata gaa tct gat gaa agt gat tat gtt     1015
Gln Val Thr Ala Thr Phe Leu Ile Glu Ser Asp Glu Ser Asp Tyr Val
310                 315                 320                 325

ggc ttt aat gca aca tat act gca ttt aac agc agt gag ctt aat aat     1063
Gly Phe Asn Ala Thr Tyr Thr Ala Phe Asn Ser Ser Glu Leu Asn Asn
                330                 335                 340

tat gag aaa att aat tgt aac ttt gag gat ggc ttt tgt ttc tgg gtc     1111
Tyr Glu Lys Ile Asn Cys Asn Phe Glu Asp Gly Phe Cys Phe Trp Val
            345                 350                 355

cag gat cta aat gat gat aat gaa tgg gaa agg att cag gga agc acc     1159
Gln Asp Leu Asn Asp Asp Asn Glu Trp Glu Arg Ile Gln Gly Ser Thr
        360                 365                 370

ttt tct cct ttt act gga ccc aat ttt gac cac act ttt ggc aat gct     1207
Phe Ser Pro Phe Thr Gly Pro Asn Phe Asp His Thr Phe Gly Asn Ala
    375                 380                 385

tca gga ttt tac att tct acc cca act gga cca gga ggg aga caa gaa     1255
Ser Gly Phe Tyr Ile Ser Thr Pro Thr Gly Pro Gly Gly Arg Gln Glu
390                 395                 400                 405
```

Fig. 3B

```
cga gtg ggg ctt tta agc ctc cct ttg gac ccc act ttg gag cca gct      1303
Arg Val Gly Leu Leu Ser Leu Pro Leu Asp Pro Thr Leu Glu Pro Ala
                410                 415                 420

tgc ctt agt ttc tgg tat cat atg tat ggt gaa aat gtc cat aaa tta      1351
Cys Leu Ser Phe Trp Tyr His Met Tyr Gly Glu Asn Val His Lys Leu
            425                 430                 435

agc att aat atc agc aat gac caa aat atg gag aag aca gtt ttc caa      1399
Ser Ile Asn Ile Ser Asn Asp Gln Asn Met Glu Lys Thr Val Phe Gln
        440                 445                 450

aag gaa gga aat tat gga gac aat tgg aat tat gga caa gta acc cta      1447
Lys Glu Gly Asn Tyr Gly Asp Asn Trp Asn Tyr Gly Gln Val Thr Leu
    455                 460                 465

aat gaa aca gtt aaa ttt aag gtt gct ttt aat gct ttt aaa aac aag      1495
Asn Glu Thr Val Lys Phe Lys Val Ala Phe Asn Ala Phe Lys Asn Lys
470                 475                 480                 485

atc ctg agt gat att gcg ttg gat gac att agc cta aca tat ggg att      1543
Ile Leu Ser Asp Ile Ala Leu Asp Asp Ile Ser Leu Thr Tyr Gly Ile
                490                 495                 500

tgc aat ggg agt ctt tat cca gaa cca act ttg gtg cca act cct cca      1591
Cys Asn Gly Ser Leu Tyr Pro Glu Pro Thr Leu Val Pro Thr Pro Pro
            505                 510                 515

cca gaa ctt cct acg gac tgt gga gga cct ttt gag ctg tgg gag cca      1639
Pro Glu Leu Pro Thr Asp Cys Gly Gly Pro Phe Glu Leu Trp Glu Pro
        520                 525                 530

aat aca aca ttc agt tct acg aac ttt cca aac agc tac cct aat ctg      1687
Asn Thr Thr Phe Ser Ser Thr Asn Phe Pro Asn Ser Tyr Pro Asn Leu
    535                 540                 545

gct ttc tgt gtt tgg att tta aat gca caa aaa gga aag aat ata caa      1735
Ala Phe Cys Val Trp Ile Leu Asn Ala Gln Lys Gly Lys Asn Ile Gln
550                 555                 560                 565

ctt cat ttt caa gaa ttt gac tta gaa aat att aac gat gta gtt gaa      1783
Leu His Phe Gln Glu Phe Asp Leu Glu Asn Ile Asn Asp Val Val Glu
                570                 575                 580

ata aga gat ggt gaa gaa gct gat tcc ttg ctc tta gct gtg tac aca      1831
Ile Arg Asp Gly Glu Glu Ala Asp Ser Leu Leu Leu Ala Val Tyr Thr
            585                 590                 595

ggg cct ggc cca gta aag gat gtg ttc tct acc acc aac aga atg act      1879
Gly Pro Gly Pro Val Lys Asp Val Phe Ser Thr Thr Asn Arg Met Thr
        600                 605                 610
```

Fig. 3C

```
gtg ctt ctc atc act aac gat gtg ttg gca aga gga ggg ttt aaa gca      1927
Val Leu Leu Ile Thr Asn Asp Val Leu Ala Arg Gly Gly Phe Lys Ala
    615                 620                 625

aac ttt act act ggc tat cac ttg ggg att cca gag cca tgc aag gca      1975
Asn Phe Thr Thr Gly Tyr His Leu Gly Ile Pro Glu Pro Cys Lys Ala
630                 635                 640                 645

gac cat ttt caa tgt aaa aat gga gag tgt gtt cca ctg gtg aat ctc      2023
Asp His Phe Gln Cys Lys Asn Gly Glu Cys Val Pro Leu Val Asn Leu
                650                 655                 660

tgt gac ggt cat ctg cac tgt gag gat ggc tca gat gaa gca gat tgt      2071
Cys Asp Gly His Leu His Cys Glu Asp Gly Ser Asp Glu Ala Asp Cys
            665                 670                 675

gtg cgt ttt ttc aat ggc aca acg aac aac aat ggt tta gtg cgg ttc      2119
Val Arg Phe Phe Asn Gly Thr Thr Asn Asn Asn Gly Leu Val Arg Phe
        680                 685                 690

aga atc cag agc ata tgg cat aca gct tgt gct gag aac tgg acc acc      2167
Arg Ile Gln Ser Ile Trp His Thr Ala Cys Ala Glu Asn Trp Thr Thr
    695                 700                 705

cag att tca aat gat gtt tgt caa ctg ctg gga cta ggg agt gga aac      2215
Gln Ile Ser Asn Asp Val Cys Gln Leu Leu Gly Leu Gly Ser Gly Asn
710                 715                 720                 725

tca tca aag cca atc ttc tct acc gat ggt gga cca ttt gtc aaa tta      2263
Ser Ser Lys Pro Ile Phe Ser Thr Asp Gly Gly Pro Phe Val Lys Leu
                730                 735                 740

aac aca gca cct gat ggc cac tta ata cta aca ccc agt caa cag tgt      2311
Asn Thr Ala Pro Asp Gly His Leu Ile Leu Thr Pro Ser Gln Gln Cys
            745                 750                 755

tta cag gat tcc ttg att cgg tta cag tgt aac cat aaa tct tgt gga      2359
Leu Gln Asp Ser Leu Ile Arg Leu Gln Cys Asn His Lys Ser Cys Gly
        760                 765                 770

aaa aaa ctg gca gct caa gac atc acc cca aag att gtt gga gga agt      2407
Lys Lys Leu Ala Ala Gln Asp Ile Thr Pro Lys Ile Val Gly Gly Ser
    775                 780                 785

aat gcc aaa gaa ggg gcc tgg ccc tgg gtt gtg ggt ctg tat tat ggc      2455
Asn Ala Lys Glu Gly Ala Trp Pro Trp Val Val Gly Leu Tyr Tyr Gly
790                 795                 800                 805

ggc cga ctg ctc tgc ggc gca tct ctc gtc agc agt gac tgg ctg gtg      2503
Gly Arg Leu Leu Cys Gly Ala Ser Leu Val Ser Ser Asp Trp Leu Val
                810                 815                 820
```

Fig. 3D

```
tcc gcc gca cac tgc gtg tat ggg aga aac tta gag cca tcc aag tgg     2551
Ser Ala Ala His Cys Val Tyr Gly Arg Asn Leu Glu Pro Ser Lys Trp
            825                 830                 835

aca gca atc cta ggc ctg cat atg aaa tca aat ctg acc tct cct caa     2599
Thr Ala Ile Leu Gly Leu His Met Lys Ser Asn Leu Thr Ser Pro Gln
        840                 845                 850

aca gtc cct cga tta ata gat gaa att gtc ata aac cct cat tac aat     2647
Thr Val Pro Arg Leu Ile Asp Glu Ile Val Ile Asn Pro His Tyr Asn
    855                 860                 865

agg cga aga aag gac aac gac att gcc atg atg cat ctg gaa ttt aaa     2695
Arg Arg Arg Lys Asp Asn Asp Ile Ala Met Met His Leu Glu Phe Lys
870                 875                 880                 885

gtg aat tac aca gat tac ata caa cct att tgt tta ccg gaa gaa aat     2743
Val Asn Tyr Thr Asp Tyr Ile Gln Pro Ile Cys Leu Pro Glu Glu Asn
                890                 895                 900

caa gtt ttt cct cca gga aga aat tgt tct att gct ggt tgg ggg acg     2791
Gln Val Phe Pro Pro Gly Arg Asn Cys Ser Ile Ala Gly Trp Gly Thr
            905                 910                 915

gtt gta tat caa ggt act act gca aac ata ttg caa gaa gct gat gtt     2839
Val Val Tyr Gln Gly Thr Thr Ala Asn Ile Leu Gln Glu Ala Asp Val
        920                 925                 930

cct ctt cta tca aat gag aga tgc caa cag cag atg cca gaa tat aac     2887
Pro Leu Leu Ser Asn Glu Arg Cys Gln Gln Gln Met Pro Glu Tyr Asn
    935                 940                 945

att act gaa aat atg ata tgt gca ggc tat gaa gaa gga gga ata gat     2935
Ile Thr Glu Asn Met Ile Cys Ala Gly Tyr Glu Glu Gly Gly Ile Asp
950                 955                 960                 965

tct tgt cag ggg gat tca gga gga cca tta atg tgc caa gaa aac aac     2983
Ser Cys Gln Gly Asp Ser Gly Gly Pro Leu Met Cys Gln Glu Asn Asn
                970                 975                 980

agg tgg ttc ctt gct ggt gtg acc tca ttt gga tac aag tgt gcc ctg     3031
Arg Trp Phe Leu Ala Gly Val Thr Ser Phe Gly Tyr Lys Cys Ala Leu
            985                 990                 995

cct aat cgc  ccc gga gtg tat gcc  agg gtc tca agg ttt  acc gaa      3076
Pro Asn Arg  Pro Gly Val Tyr Ala  Arg Val Ser Arg Phe  Thr Glu
        1000                1005                1010

tgg ata caa  agt ttt cta cat tag cgcatttctt aaactaaaca ggaaagtcgc   3130
Trp Ile Gln  Ser Phe Leu His
        1015
```

Fig. 3E

```
attattttcc cattctactc tagaaagcat ggaaattaag tgtttcgtac aaaaatttta   3190
aaaagttacc aaaggttttt attcttacct atgtcaatga aatgctaggg ggccagggaa   3250
acaaaatttt aaaaataata aaattcacca tagcaataca gaataacttt aaaataccat   3310
taaatacatt tgtatttcat tgtgaacagg tatttcttca cagatctcat ttttaaaatt   3370
cttaatgatt atttttatta cttactgttg tttaaaggga tgttatttta aagcatatac   3430
catacactta agaaatttga gcagaattta aaaaagaaag aaaataaatt gtttttccca   3490
aagtatgtca ctgttggaaa taaactgcca taaattttct agttccagtt tagtttgctg   3550
ctattagcag aaactcaatt gtttctctgt cttttctatc aaaattttca acatatgcat   3610
aaccttagta ttttcccaac caatagaaac tatttattgt aagcttatgt cacaggcctg   3670
gactaaattg attttacgtt cctctt                                       3696
```

Fig. 3F

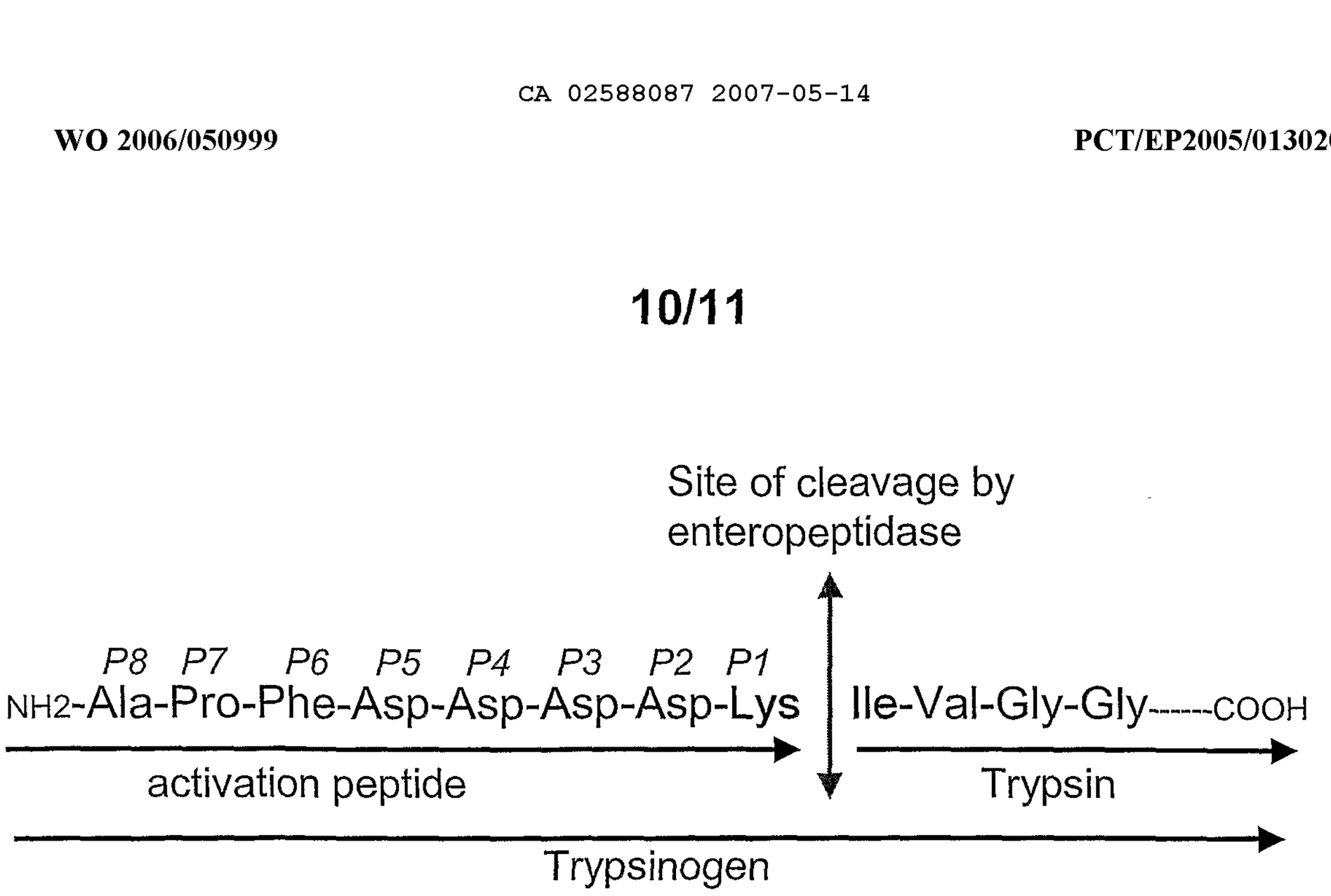

Site of cleavage by enteropeptidase
P8 P7 P6 P5 P4 P3 P2 P1
NH2-Ala-Pro-Phe-Asp-Asp-Asp-Asp-Lys
Ile-Val-Gly-Gly------COOH
activation peptide
Trypsin
Trypsinogen

Fig. 4

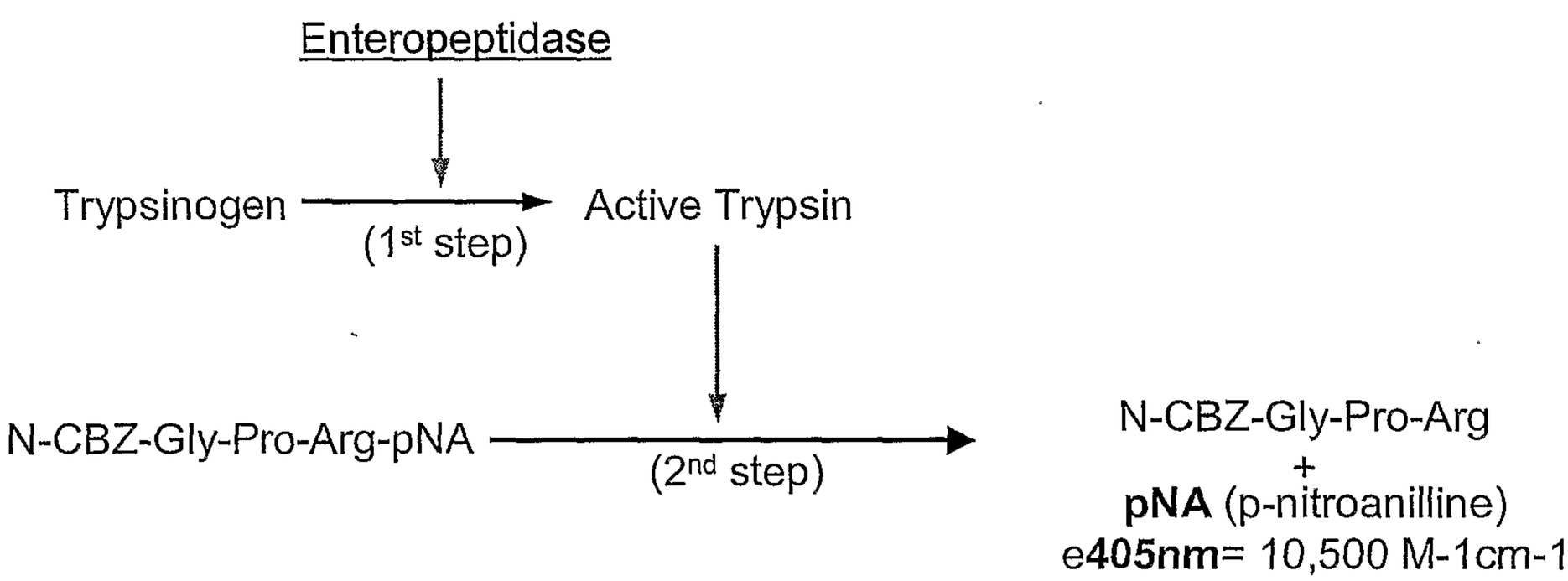
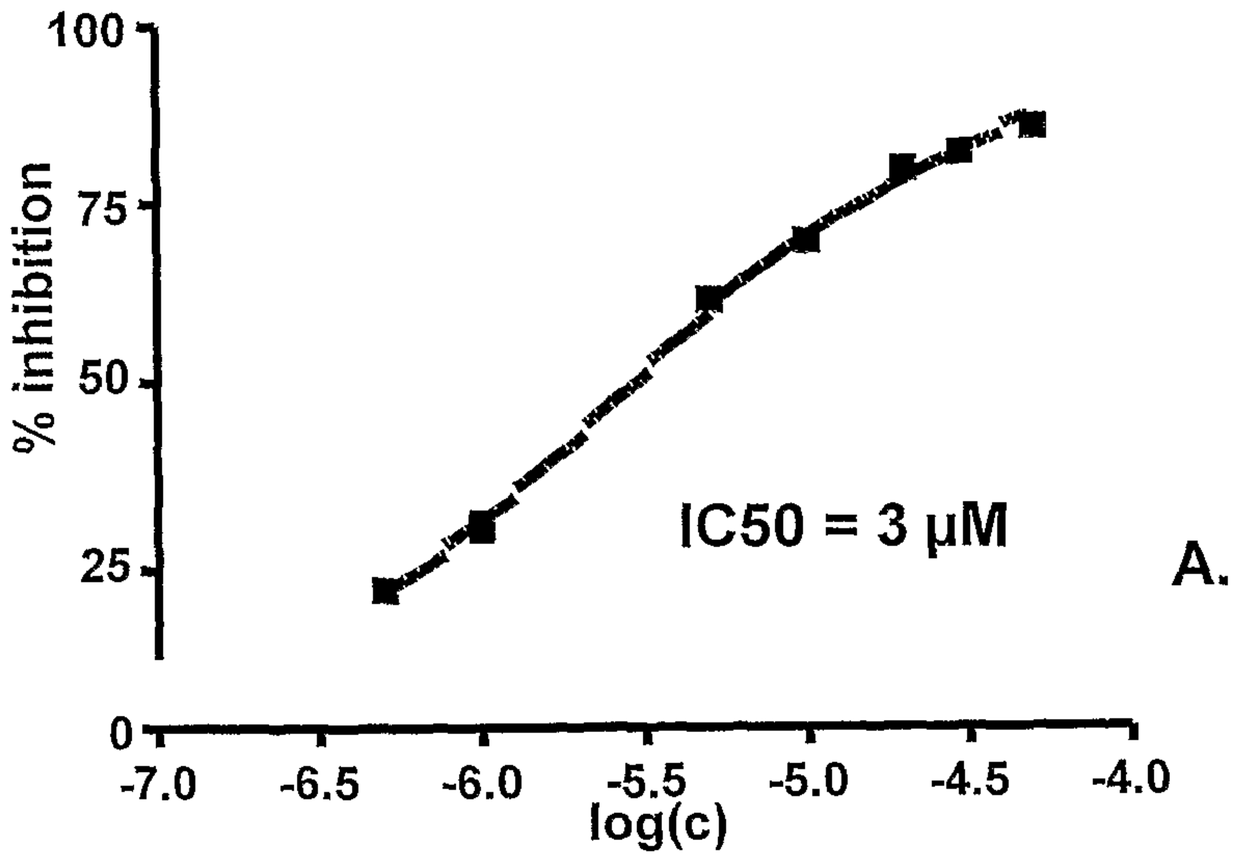
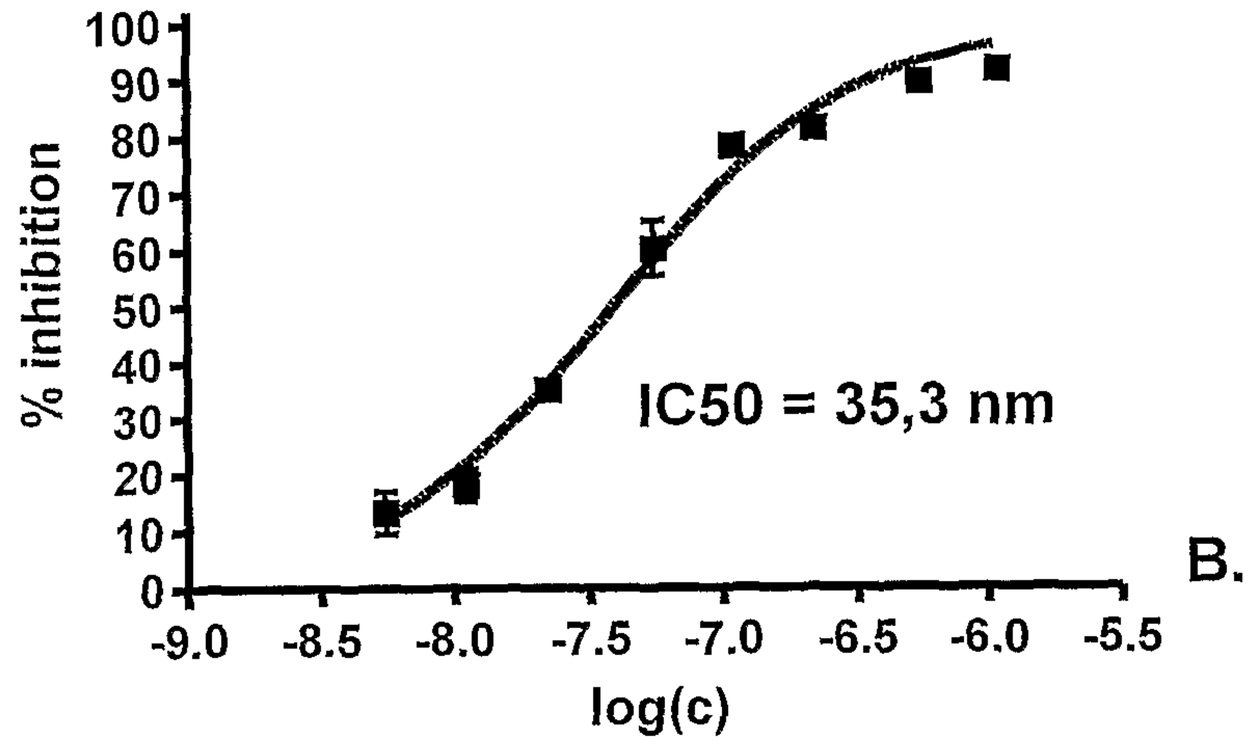

Enteropeptidase
Trypsinogen
(1st step)
Active Trypsin
N-CBZ-Gly-Pro-Arg-pNA
(2nd step)
N-CBZ-Gly-Pro-Arg
+
pNA (p-nitroanilline)
e405nm= 10,500 M-1cm-1

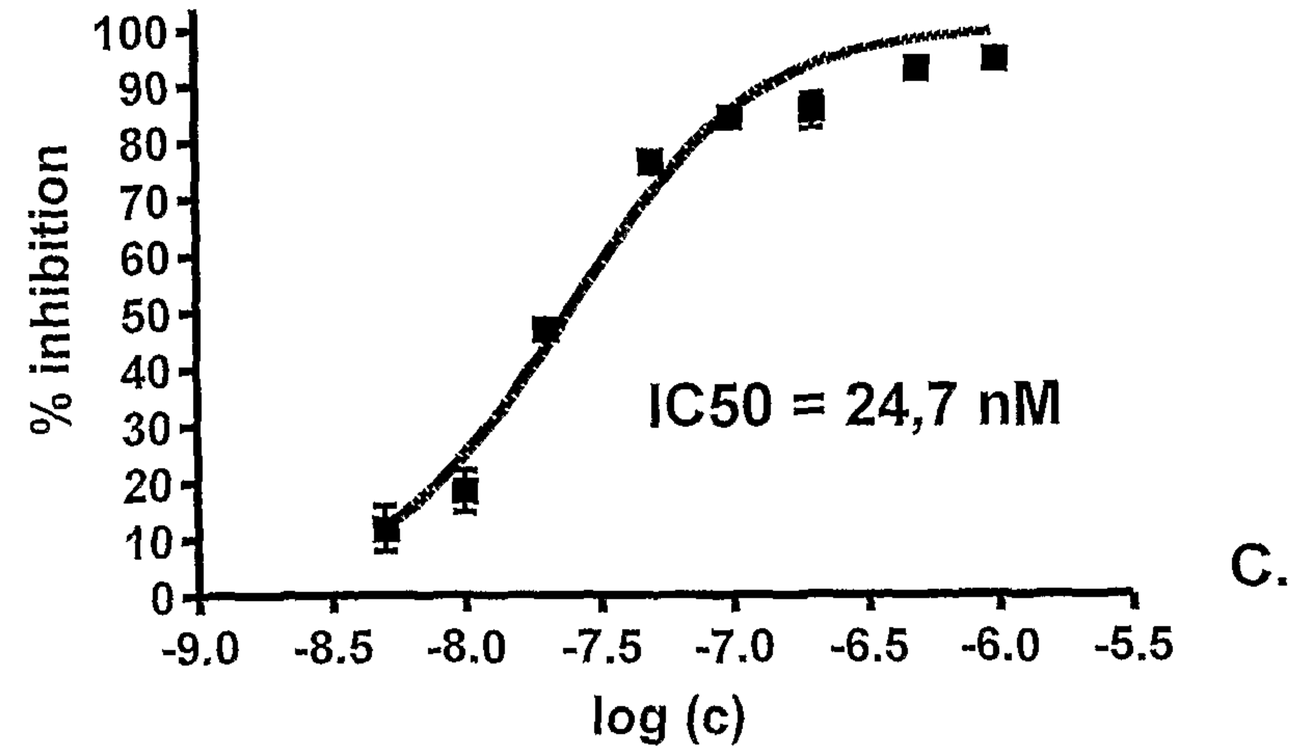
Fig. 5


100
75
50
25
0
% inhibition
IC50 = 3 µM
-7.0
-6.5
-6.0
-5.5
-5.0
-4.5
-4.0
log(c)

A.

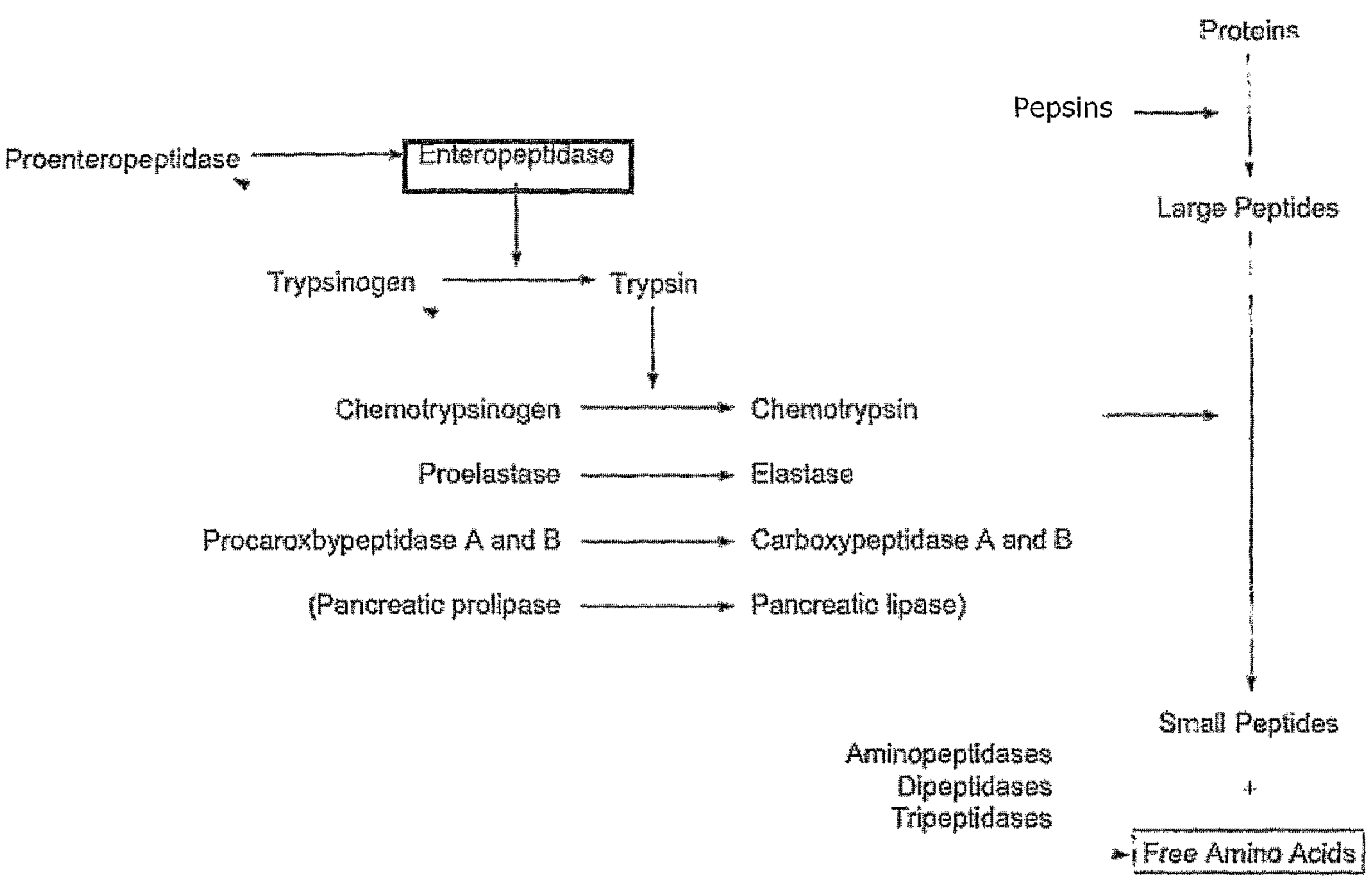

100
90
80
70
60
50
40
30
20
10
0
% inhibition
IC50 = 35,3 nm
-9.0
-8.5
-8.0
-7.5
-7.0
-6.5
-6.0
-5.5
log(c)

B.


100
90
80
70
60
50
40
30
20
10
0
% inhibition
IC50 = 24,7 nM
-9.0
-8.5
-8.0
-7.5
-7.0
-6.5
-6.0
-5.5
log (c)

C.

Fig. 6


Proteins
Pepsins
Large Peptides
Proenteropeptidase
Enteropeptidase
Trypsinogen
Trypsin
Chemotrypsinogen
Chemotrypsin
Proelastase
Elastase
Procaroxbypeptidase A and B
Carboxypeptidase A and B
(Pancreatic prolipase
Pancreatic lipase)
Small Peptides
Aminopeptidases
Dipeptidases
Tripeptidases
Free Amino Acids