(54) AGGREGATE-FREE URATE OXIDASE FOR PREPARATION OF NON-IMMUNOGENIC POLYMER CONJUGATES

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(57) ABSTRACT
A naturally occurring or recombinant protein, especially a mutein of porcine urate oxidase (uricase), that is essentially free of large aggregates can be rendered substantially non-immunogenic by conjugation with a sufficiently small number of strands of polymer such that the bioactivity of the protein is essentially retained in the conjugate. Such conjugates are unusually well suited for treatment of chronic conditions because they are less likely to induce the formation of antibodies and/or accelerated clearance than are similar conjugates prepared from protein preparations containing traces of large aggregates.

40 Claims, 6 Drawing Sheets
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Size-Exclusion HPLC on Superdex 200 of Unfractionated PKS Uricase (Load) and Mono Q Column Fractions in the Low-Salt Pool

**FIG. 2**
Size-Exclusion HPLC on Superdex 200 of Mono Q
Column Fractions of PKS Uricase in the High-Salt Pool

FIG. 3
FIG. 4

Octamer Content of Mono Q Column Fractions of PKS Uricase

- Octamer Content of the Load
- Low-Salt Pool
- High-Salt Pool

Legend:
- □ by Absorbance at 276 nm
- □ by Light Scattering at 90 degrees
FIG. 5

UV Uricase Assays of Sera from Mice Injected with 6 x 10-kDa PEG Conjugates of PKS Uricase or of Pools from Mono Q Column Fractions
(Mice Were Bled 24 Hours after Each Weekly Injection.)

Data for the Low-Salt and High-Salt Pools were shifted on the x-axis by 0.1 and 0.2 units, respectively.
FIG. 6

6x10-kDa PEG Conjugates of PKS Uricase:
Unfractionated and Low-Salt or High-Salt Pools

- Low-Salt Pool
- Unfractionated PKS Uricase
- High-Salt Pool

IgG Peroxidase Values, mAU/min

Week Number

Mouse 1  Mouse 2  Mouse 3  Mouse 4  Mouse 5  Mouse 6  Mouse 7  Mouse 8
AGGREGATE-FREE URATE OXIDASE FOR PREPARATION OF NON-IMMUNOGENIC POLYMER CONJUGATES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 10/928,370, filed Aug. 30, 2004, which is a continuation of U.S. application Ser. No. 09/501,730, filed Feb. 10, 2000 (now U.S. Pat. No. 6,783,965) and is a continuation-in-part of U.S. application Ser. No. 09/839,946, filed Apr. 19, 2001 (now U.S. Pat. No. 7,573,089), which is a divisional of U.S. application Ser. No. 09/370,084, filed Aug. 6, 1999 (now U.S. Pat. No. 6,576,235), which claims the benefit of U.S. Provisional Appl. No. 60/219,318, filed Aug. 6, 1998 (now expired), the disclosures of all of which applications are incorporated herein by reference in their entireties.

STATEMENT OF GOVERNMENT RIGHTS IN THE INVENTION

A portion of the research described in this application was made with support from the U.S.-Israel Binational Industrial Research and Development Foundation. Accordingly, the U.S. Government may have certain rights in the invention.

REFERENCE TO A SEQUENCE LISTING

SUBMITTED ELECTRONICALLY VIA EFS-WEB

The content of the electronically submitted sequence listing (Name: Substitute Sequence Listing.ascii.txt; Size: 8,639 bytes; and Date of Creation: Jun. 5, 2009) is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to purification and chemical modification of proteins to prolong their circulating lifetimes and reduce their immunogenicity. More specifically, the invention relates to the removal of aggregates larger than octamers from urate oxidases (uricases) prior to conjugation of poly(ethylene glycols) or poly(ethylene oxides). This substantially eliminates uricase immunogenicity without compromising its uricolytic activity.

2. Description of the Related Art

Statements contained in this background section do not constitute an admission of prior art, but instead reflect the inventors’ own subjective comments on and interpretations of the state of the art at the time the invention was made. These interpretations may include personal, heretofore undisclosed, insights of the inventors, which insights were not themselves incorporated part of the prior art.

Urate oxidases (uricases; E.C. 1.7.3.3) are enzymes that catalyze the oxidation of uric acid to a more soluble product, allantoin, a purine metabolite that is more readily excreted. Humans do not produce enzymatically active uricase, as a result of several mutations in the gene for uricase acquired during the evolution of higher primates. Wu, X., et al., (1992) J Mol Evol 34:78-84. As a consequence, in susceptible individuals, excessive concentrations of uric acid in the blood (hyperuricemia) and in the urine (hyperuricosuria) can lead to painful arthritis (gout), disfiguring urate deposits (tophi) and renal failure. In some affected individuals, available drugs such as allopurinol (an inhibitor of uric acid synthesis) produce treatment-limiting adverse effects or do not relieve these conditions adequately. Hande, K R, et al., (1984) Am J Med 76:47-56; Fam, AG, (1990) Bailliere’s Clin Rheumatol 4:177-192. Injections of uricase can decrease hyperuricemia and hyperuricosuria, at least transiently. Since uricase is a foreign protein in humans, however, even the first injection of the unmodified protein from Aspergillus flavus has induced anaphylactic reactions in several percent of treated patients (Pui, C-H., et al., (1997) Leukemia 11:1813-1816), and immunologic responses limit its utility for chronic or intermittent treatment. Donadio, D., et al., (1981) Nouv Presse Méd 10:711-712; Leautie, M., et al., (1983) Rev Rhum Mal Ostéoartic. 50:553-554.

U.S. patent application Ser. No. 09/370,084 (now U.S. Pat. No. 6,783,235) and published International Application No. PCT/US99/17514 (now abandoned), the entire contents of which are incorporated herein by reference, disclose poly(ethylene glycol) uricase (PEG-uricase) that retains at least about 75% of the uricolytic activity of unconjugated uricase and has substantially reduced immunogenicity. In one such purified uricase, each subunit is covalently linked to an average of 2 to 10 strands of PEG, wherein each molecule of PEG may have a molecular weight between about 5 kDa and 100 kDa.

The aggregation of proteins is known to increase their immunogenicity. This understanding has contributed to the development of methods for intentionally aggregating proteins by treatments such as thermal denaturation and cross-linking by exposure to glutaraldehyde prior to use in the preparation of vaccines or for immunization of animals to produce antisera.

Unintentional aggregation of proteins has also been recognized as contributing to immunization or sensitization during clinical use of therapeutic proteins, e.g. for human gamma globulin (Henney et al., (1968) N. Engl. J. Med. 278:2244-2246) and for human growth hormone (Moore et al., (1980) J. Clin. Endocrinol. Metab. 51:691-97). The contribution of aggregates to the immunogenicity of human interferon alpha has been demonstrated in BALB/c mice (Braun et al. (1997) Pharm. Res. 14:1472-1478) and an enzyme-linked immunosorbent assay (ELISA) has been developed for their measurement (Braun et al. (1997) Pharm. Res. 14:1394-1400).

In contrast to the known effects of aggregation on the immunogenicity of proteins, however, reports of the effect of aggregation on the immunogenicity of proteins conjugated to poly(alkylene glycols) such as PEG. There is a need for poly(alkylene glycol)-urate conjugates that substantially eliminates uricase immunogenicity without compromising its uricolytic activity. The present invention provides such compositions.

SUMMARY OF THE INVENTION

Conjugation of proteins with poly(alkylene glycols), especially PEG, produces conjugates with reduced immunogenicity and increased persistence in the bloodstream. In attempts to produce substantially non-immunogenic conjugates of uricase that retain substantially all of the uricolytic activity of the unmodified uricase, it was discovered that traces of large aggregates of uricase in the starting material were surprisingly effective at provoking both antibody formation and accelerated clearance from the circulation, both of which are deleterious, after repeated injections of PEG conjugates from uricase containing such aggregates. Surprisingly, the present inventors found that the increased immunogenicity and accelerated clearance were not due to the presence of well-defined, moderate-sized aggregates of the uricase subunit that are larger than the native tetramer, e.g.
aggregates containing eight subunits (octamers). The octameric form of uricase is present at sufficiently high concentrations in most preparations of uricase to be detectable by its absorbance of UV light, e.g. at 214 nm or 276 nm, or by its contribution to the refractive index or other measurements of protein concentration. Nevertheless, the octamers themselves were found to contribute minimally to the immunogenicity and accelerated clearance of PEG-uricase conjugates, in contrast with the much smaller quantities of the much larger aggregates that are undetectable by UV absorbance under the conditions tested but are readily detected by static (Raleigh) or dynamic light scattering. Therefore, the removal of such traces of very large aggregates prior to conjugation with PEG was found to decrease the immunogenicity and the accelerated clearance of the resultant PEG-uricase conjugates to a surprising extent.

One preferred embodiment of the present invention is a purified urate oxidase (uricase) substantially free of aggregates larger than octamers. Preferably, the uricase is mammalian uricase. More preferably, the uricase is porcine liver, bovine liver or ovine liver uricase. In one aspect of this preferred embodiment, the uricase has substantially the sequence of porcine, bovine or baboon liver uricase. Advantageously, the uricase is a recombinant enzyme having substantially the sequence of one of said uricases. Alternatively, the uricase is an invertebrate uricase. Preferably, the uricase comprises an amino terminus and a carboxy terminus, and wherein the uricase is truncated at one terminus or both termini. Advantageously, the uricase is a fungal or microbial uricase. Preferably, the fungal or microbial uricase is isolated from Aspergillus flavus, Arthrobacter globiformis, Bacillus sp. or Candida utilis, or is a recombinant enzyme having substantially the sequence of one of said uricases. Alternatively, the uricase is an invertebrate uricase. Preferably, the invertebrate uricase is isolated from Drosophila melanogaster or Drosophila pseudoobscura, or is a recombinant enzyme having substantially the sequence of one of said uricases. In another aspect of this preferred embodiment, the uricase is a plant uricase. Preferably, the plant uricase is isolated from root nodules of Glycine max or is a recombinant enzyme having substantially the sequence of the uricase.

In one aspect of this preferred embodiment, the uricase described above is conjugated to poly(ethylene glycol) or poly(ethylene oxide), under conditions such that the uricase in the conjugate is substantially free of aggregates larger than octamers. Preferably, the uricase is conjugated to poly(ethylene glycol) or poly(ethylene oxide) via a urethane (carbamate), secondary amine or amide linkage. In one aspect of this preferred embodiment, the poly(ethylene glycol) is branched.

The present invention also provides a pharmaceutical composition for lowering uric acid levels in a body fluid or tissue, comprising the uricase conjugate described above and a pharmaceutically acceptable carrier. Preferably, the composition is stabilized by lyophilization and dissolves upon reconstitution to provide solutions suitable for parenteral administration.

Another embodiment of the invention is a method for purifying uricase having reduced immunogenicity, comprising the step of separating uricase aggregates larger than octamers in uricase fractions, and excluding such aggregates from the purified uricase. Preferably, the separating step comprises the step of detecting aggregates larger than octamers from at least a portion of the uricase fractions and excluding the fractions containing the aggregates. Advantageously, the detecting step comprises measurement of light scattering.

The present invention also provides isolated uricase prepared by the method described above.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates uricase activity, total protein and salt concentrations in fractions from a Pharmacia Biotech Mono Q column (1x10 cm) anion exchange column. Uricase activity was measured at room temperature by monitoring the decrease in absorbance at 292 nm of 100 μM uric acid in 200 mM sodium borate, pH 9.2. Total protein was determined from the area under the curve of the absorbance peak of uricase in size-exclusion HPLC analyses. Salt concentrations were calculated from the conductivities at room temperature using a standard curve for NaCl in the same buffer.

FIG. 2 illustrates size-exclusion HPLC analysis on a Pharmacia Superdex 200 column (1x30 cm) of the load and selected fractions from a preparative Mono Q column chromatography of porcine uricase (containing the mutations R291K and T301S (PKS uricase)) showing data obtained by a light-scattering detector at 90° C. (upper curves) and by absorbance at 276 nm (lower curves). The different signal strengths of the tetrameric, octameric and more highly aggregated forms of uricase in the unfractionated sample (load) and the various fractions are evident. The load was diluted 1/3 with Mono Q column buffer, fraction 5 was diluted 1/3 and fraction 6 was diluted 1/6. Fractions 5 and 6 were combined to form the "low salt pool.”

FIG. 3 illustrates size-exclusion analyses of fractions from the Mono Q column in FIG. 1, showing data obtained by a light-scattering detector at 900 and by absorbance at 276 nm, as in FIG. 2. The fractions shown in this figure were used to form the "high salt pool," from which PEG conjugates were prepared and injected into BALB/c mice. The resultant serum activities and immunologic responses in BALB/c mice are shown in FIGS. 5 and 6.

FIG. 4 illustrates octamer content, determined by absorbance at 276 nm and by light scattering at 90°, calculated from the data in FIGS. 2 and 3, of unfractionated PKS uricase and of selected fractions from the preparative MonoQ column chromatography of PKS uricase (FIG. 1).

FIG. 5 illustrates TV assays, as in FIG. 1, of uricase activity after a 4-hour incubation at 37° C., in sera drawn 24 hours after each of six weekly injections of 6x10-kDa PEG conjugates of PKS uricase or of pools from Mono Q column fractions.

FIG. 6 illustrates ELISA analyses of IgG antibody formation against PEG conjugates of PKS uricase and against PEG.
conjugates of the pools of fractions from the Mono Q column shown in FIG. 1 in sera drawn 24 hours after each of six weekly injections of female BALB/c mice with 0.2 mg of uricase protein per 20 grams of body weight. For each mouse, data from bleedings 24 hours after the first through sixth injections are shown from left to right. The assay conditions are described in Example 6. Data for the eight mice in each group were arranged in order of increasing immune response, from left to right.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Previous studies have shown that when a significant reduction in the immunogenicity and/or antigenicity of uricase is achieved by conjugation with PEG (PEGylation), it is invariably associated with a substantial loss of uricolytic activity. The present invention includes the observation that traces of aggregates of urate oxidases larger than octamers substantially contribute to immunogenicity and the induction of accelerated clearance of PEG-uricase conjugates. This discovery is most likely applicable to proteins other than uricases, including interferons and growth factors.

The safety, convenience and cost-effectiveness of biopharmaceuticals are all adversely impacted by decreases in their potencies and the resultant need to increase the administered dose. Thus, there is a need for a safe and effective alternative means for lowering elevated levels of uric acid in body fluids, including blood and urine. The present invention provides a method for producing uricase that excludes uricase aggregates larger than octamers for use in the synthesis of PEG-uricase. This PEG-uricase retains all or nearly all of the uricolytic activity of the unmodified enzyme. The present invention also provides purified uricase substantially free of aggregates larger than octamers. The term "substantially free" indicates that the purified uricase comprises no more than about 2%, and preferably no more than about 1% of aggregates larger than octamers.

The present invention provides a method for purifying uricase such that aggregates larger than octamers are excluded from the purified preparation. Because these larger aggregates are highly immunogenic, their presence in the purified uricase preparation is undesirable. The method involves monitoring column fractions by light scattering rather than or in addition to ultraviolet absorbance at 280 nm, because the aggregates may be too dilute to be detected by ultraviolet absorbance. The purified uricase is then conjugated to water-soluble polymers, preferably poly(ethylene glycols) or poly(ethylene oxides) as described in copending U.S. application Ser. No. 09/370,084, the entire contents of which are incorporated herein by reference.

The removal of aggregated uricase from a preparation consisting predominantly of tetrameric uricase can be accomplished by any of the methods known to those skilled in the art, including size-exclusion chromatography, ion-exchange chromatography, ultrafiltration through a microporous membrane and centrifugation, including ultracentrifugation. The separation method may include separation and analysis of fractions and the rejection or exclusion of those fractions containing excessive quantities of large aggregates. The resultant uricase preparation is better suited for the synthesis of substantially non-immunogenic conjugates of uricase than is the unfractonated uricase. For chronic administration, it is important that PEG conjugates of proteins, e.g. PEG-uricase, have low immunogenicity and do not provoke progressively more rapid clearance from the bloodstream after repeated doses.

The invention also provides pharmaceutical compositions of the polymer-uricase conjugates. These conjugates are substantially non-immunogenic and retain at least 75%, preferably 85%, and more preferably 95% or more of the uricolytic activity of the unmodified enzyme. Uricases suitable for conjugation to water-soluble polymers include naturally occurring urate oxidases isolated from bacteria, fungi and the tissues of plants and animals, both vertebrates and invertebrates, as well as recombinant forms of uricase, including mutated, hybrid, and/or truncated enzymatically active variants of uricase. Water-soluble polymers suitable for use in the present invention include linear and branched poly(ethylene glycols) or poly(ethylene oxides), all commonly known as PEGs. Examples of branched PEG are the subject of PCT or US applications.

One embodiment of the present invention is a conjugate of urate oxidase (uricase) that retains at least about 75% of the uricolytic activity of unconjugated uricase and has substantially reduced immunogenicity. The uricase of this aspect of the invention may be recombinant. Whether recombinant or not, the uricase may be of mammalian origin. In one aspect of this embodiment, the uricase may be porcine, bovine or ovine liver uricase. In another aspect of this embodiment, the uricase may be chimeric. The chimeric uricase may contain portions of porcine liver and/or baboon liver uricase. For example, the chimeric uricase may be porcine uricase containing the mutations R291K and T301S (PKS uricase).

Alternatively, the uricase may be baboon liver uricase in which tyrosine 97 has been replaced by histidine, whereby the specific activity of the uricase may be increased by at least about 60%. The uricase of the invention, whatever the origin, may also be in a form that is truncated, either at the amino terminal, or at the carboxyl terminal, or at both terminals. Likewise, the uricase may be fungal or microbial uricase.

In one aspect of this embodiment, the fungal or microbial uricase may be a naturally occurring or recombinant form of uricase from Aspergillus flavus, Arthrobacter globiformis, Bacillus sp. or Candida utilis. Alternatively, the uricase may be an invertebrate uricase, such as, for example, a naturally occurring or recombinant form of uricase from Drosophila melanogaster or Drosophila pseudoobscura. The uricase of the invention may also be a plant uricase, for example, a naturally occurring or recombinant form of uricase from soybean root nodule (Glycine max). The PEG may have an average molecular weight between about 5 kDa and 100 kDa; preferably the PEG may have an average molecular weight between about 8 kDa and 60 kDa; more preferably, the PEG may have an average molecular weight between about 5 kDa and about 40 kDa, such as, for example, 10 to 20 kDa. The average number of covalently coupled strands of PEG may be 2 to 12 strands per uricase subunit; preferably, the average number of covalently coupled strands may be 6 to 10 per subunit; more preferably, the average number of strands of PEG may be 7 to 9 per subunit. In one aspect of this embodiment, the uricase may be tetrameric. The strands of PEG may be covalently linked to uricase via urethane (carbamate) linkages, secondary amine linkages, and/or amide linkages. When the uricase is a recombinant form of any of the uricases mentioned herein, the recombinant form may have substantially the sequence of the naturally occurring form.

One preferred mammalian uricase is recombinant pig-baboon chimeric uricase, composed of portions of the sequences of pig liver and baboon liver uricase, both of which were first determined by Wu, et al., (1989). One example of
such a chimeric uricase contains the first 288 amino acids from the porcine sequence (SEQ ID NO: 1) and the last 16 amino acids from the baboon sequence (SEQ ID NO: 2). Since the latter sequence differs from the porcine sequence at only two positions, having a lysine (K) in place of arginine at residue 291 and a serine (S) in place of threonine at residue 301, this mutant is referred to as pig-K-S or PKS uricase (SEQ ID NO: 3). PKS uricase has one more lysine residue and, hence, one more potential site of PEGylation than either the porcine or baboon sequence.


In one embodiment of the invention, uricase may be conjugated via a biologically stable, nontoxic, covalent linkage to 25 Applications for DNA Amplification. method of synthesis of NPC-PEG is described by

By incubating uricase in the presence of the succinimidyl aldehyde (Shearwater Polymers) and sodium cyanoborohydride. In conjugates containing PEG with a molecular weight of 10 kDa, the maximum number of strands of PEG that were coupled per subunit, while retaining at least 75% of the uricolytic activity of the unmodified enzyme, was about 12 strands for mammalian uricases (e.g. PKS uricase, a mutein of porcine uricase) and the enzyme from thermophilic Bacillus sp.

In another preferred embodiment, substantially all large aggregates of the enzyme may be removed by ion-exchange chromatography (FIGS. 1-3) or size-exclusion chromatography at a pH between about 9 and 10.5, preferably 10.2, prior to conjugation of the resulting substantially aggregate-free preparation of uricase to PEG. The molecular weight of the uricase in each fraction from the preparative column may be monitored by any size-dependent analytical technique, including, for example, HPLC, conventional size-exclusion chromatography, centrifugation, light scattering, capillary electrophoresis or gel electrophoresis in a non-denaturing buffer. For aggregate-free uricase isolated using size-exclusion chromatography, fractions containing only the 140-kDa and 280-kDa forms of the enzyme may be pooled and used for conjugation to PEG. For tetrameric plus octameric uricase isolated using ion-exchange chromatography, fractions from the ion-exchange column may be analyzed with respect to size to determine which fractions contain substantial amounts of the tetrameric and octameric forms without the large aggregate...
transplantation and malignant disease. PEG-uricase conju­
gates prepared from uricase that is essentially free of large aggregates (detectable by light scattering) could be re­newed at least six times at one-week intervals with much less ev­i
dence of accelerated clearance rates (FIG. 5) and without the detectable formation of antibodies, as measured by a sensitive enzyme-linked immunosay (FIG. 6). The use of highly purified tetrameric or octameric uricase further distinguishes the improved conjugates of the present invention from the 15
tional depot formulations comprise bioerodible or biodegrad­ing PEG-uricase comprises the method disclosed in U.S. Pat. No. 5,653,974, which is hereby incorporated by reference. The use of bioerodible, biodegradable and other depot for­mulations is expressly contemplated in the present invention. The use of infusion pumps and matrix entrapment systems for delivery of PEG-uricase is also within the scope of the present invention. PEG-uricase may also advantageously be enclosed in micelles or liposomes. Liposome encapsulation technol­gy is well known in the art. See, e.g., Lasic, D., et al., (Eds.) (1995) Stealth Liposomes. Boca Raton, Fla.: CRC Press.

The PEG-uricase pharmaceutical compositions of the invention will decrease the need for hemodialysis in patients at high risk of urate-induced renal failure, e.g., organ trans­plant recipients (see Venkataseshan, V, S, et al., (1990) Ne­phron 56:317-321) and patients with some malignant diseases. In patients with large accumulations of crystalline urate (tophi), such pharmaceutical compositions will improve the quality of life more rapidly than currently available treat­ments.

The following examples, which are not to be construed as limiting the invention in any way, illustrate the various aspects disclosed above. These examples describe PEG­uricase preparations prepared by coupling activated PEG (e.g., the p-nitrophenyl carbonate derivative) to a mutein of porcine uricases. These examples provide guidance to one of ordinary skill in the art for producing substantially non-immunogenic conjugates of uricase that retain at least about 75% of the uricolytic activity of the unmodified enzyme and are well suited for chronic administration.

Example 1

Preparative Ion-Exchange Chromatography of Uricase

Preparative ion-exchange chromatography was performed on a Fast Protein Liquid Chromatography (FPLC) apparatus (Amersham Pharmacia, Piscataway, N.J.). The Mono Q column (10x1 cm, Amersham Pharmacia) was eluted with a gradient of 50 mM sodium carbonate, pH 10.3, 0.1 M NaCl (Buffer A) to 50 mM sodium carbonate, pH 10.3, 0.6 M NaCl (Buffer B) at a flow rate of 0.5 mL/min except that the sample was loaded at a lower flow-rate. This technique was used to fractionate 25 mL of a solution of PKS uricase (pH 10.3). PKS uricase was obtained from Bio-Technology General Limited (Rehovot, Israel). The latter is recombinant porcine uricase in which one residue of lysine (K) and one residue of serine (S) have replaced one residue of arginine and one residue of threonine, respectively, in the parental porcine sequence (Lee et al. (1988) Science 239:1288-1291, Wu et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:9412-9416). After the sample was loaded, the column was washed with 100 mL of Buffer A. The peak of uricase began to elute at the end of a 31-mL linear gradient of 0 to 26% Buffer B. Most of the uricase was eluted isocratically by 7 mL of buffer containing 26% Buffer B. The remainder of the recovered uricase was eluted by a linear 89-mL gradient of 26% to 100% buffer B. Fractions of 4 mL or 6 mL were collected. Aliquots of 75% of the Fraction #4-11 were assayed for uricase, total protein and NaCl concentration (FIG. 1) and were analyzed by size-exclusion high performance liquid chromatography (HPLC) as described in Example 2 (FIGS. 2 and 3). The remaining portions of Fractions #5-10 were coupled to PEG, as

Example 2

Liposome Encapsulation of Uricase

The uricase in Fraction #10 was subjected to lipid encapsulation. Mammalian cells, porcine kidney tissue cultures, or tissue culture cell lines were either prepared and used directly or the uricase solution was added to the culture medium in which the cells were grown. After a period of time, the cells were harvested and the uricase solution was removed. The uricase solution was either added to the culture medium with the uricase still in the medium or the uricase solution was added directly to the cells so that the uricase was in contact with the cells. Uricase preparations used by some previous investigators may have led them to couple large numbers of strands of PEG in 15

Example 3

Micelle Encapsulation of Uricase

The uricase in Fraction #10 was subjected to micelle encapsulation. Mammalian cells, porcine kidney tissue cultures, or tissue culture cell lines were either prepared and used directly or the uricase solution was added to the culture medium in which the cells were grown. After a period of time, the cells were harvested and the uricase solution was removed. The uricase solution was either added to the culture medium with the uricase still in the medium or the uricase solution was added directly to the cells so that the uricase was in contact with the cells. Uricase preparations used by some previous investigators may have led them to couple large numbers of strands of PEG in
described in Example 3. Based on the results of the analyses in Example 2, the PEG conjugates of Fractions #5 and 6 were combined as the “Low-Salt Pool” and the PEG conjugates of Fractions #7-10 were combined as the “High-Salt Pool,” as indicated in FIG. 1.

Example 2

Size-Exclusion Chromatography of Uricase
Monitored by Light Scattering and Ultraviolet Absorbance

Size-exclusion HPLC was performed at room temperature on a Superdex 200 column (1 x 30 cm, Amersham Pharmacia Biotech) on unfraccionated PKS uricase and on selected fractions from the preparative Mono Q chromatography of PKS uricase of Example 1. The elute from the absorbance monitor (UV 2000) of the Thermo Separations HPLC (Sunnyvale, Calif.) was analyzed by light scattering at 90° to the incident light, using a MiniDawn detector from Wyatt Technologies (Santa Barbara, Calif.).

The results shown in FIGS. 2-4 illustrate the resolution among the tetramer, octamer and larger aggregates of the uricase subunit and the different proportions of the signals detected from these forms of uricase in the various samples. Unlike the absorbance signal, which is directly proportional to the concentration, the light scattering signal is proportional to the product of the concentration times the size of the light scattering unit. The resultant sensitivity of the light scattering detector to very small amounts of highly aggregated uricase revealed the presence of the largest aggregates, which are eluted at or near the void volume (approximately 7 mL).

Example 3

Synthesis of PEG-Uricase Conjugates

Unfraccionated PKS uricase (from Bio-Technology General Limited) and the uricase in fractions from the Mono Q column of Example 1 were coupled to 10-kDa PEG using the p-nitrophenyl carbonate derivative of PEG (NPC-PEG) obtained from Shearer ater Polymers (Huntsville, Ala.). The preparation of NPC-PEG from PEG using phenylchloroformates has been described in several reports (e.g. Veronese, F M, et al., (1985) Appl Biochem Biotechnol 11:141-152; Kito, M, et al., (1996) J Clin Biochem Nutr 21:101-111) and NPC-PEG has been used for the synthesis of PEG-protein conjugates by previous investigator, including the present inventors (e.g. Veronese et al., supra; Sherman, M R, et al., in J M Harris, et al., (Eds.) Poly(ethylene glycol) Chemistry and Biological Applications, ACS Symposium Series 680 (pp. 155-176) Washington, D.C.: American Chemical Society). The number of strands of 10-kDa PEG coupled to each subunit of uricase was determined to be six by the method described by Kunitani, M, et al., (1991) J Chromatogr 588:125-137.

Example 4

In Vivo Serum Persistence and Immunogenicity of Uricase and PEG-Uricase

PEG conjugates of recombinant mammalian uricases, prepared according to the method of Example 3, were adjusted to 1 mg protein/mL in phosphate-buffered saline (PBS), pH 7.4, for injection. Samples were frozen and stored until analyzed or injected. Samples were warmed to 37° C. for up to 1 hour prior to injection into groups of eight BALB/c female mice. The groups of mice had mean weights in the range of 18-22 g at the start of the studies. The weights of all mice were monitored and evidence of adverse reactions to the injections or other evidence of ill health was recorded. Twenty-four hours after each of six weekly injections, the animals were anesthetized with ketamine and 100-200 μL of blood was obtained retro-orbitally, except at sacrifice (exsanguination), when a larger volume was collected. Serum was prepared from blood that had clotted for between 4 and 32 hours at 2-8° C. Sera were stored at −20° C. Sera were analyzed for uricolytic activity as described in Example 5 and analyzed for antibodies against uricases as described in Example 6.

Example 5

Uricolytic Activity Assays of PEG-Uricase in Sera from Mice Injected with PEG-Uricase

An activity assay based on ultraviolet light absorbance (UV assay) was performed with 100 μM uric acid as the substrate in 200 mM sodium borate, pH 9.2, in a microplate adaptation of the method of I. Fridovich (J Biol Chem. (1965) 240:2491-2494). The decrease in absorbance at 292 nm was monitored for 15 minutes at room temperature in a 96-well plate with a UV-transparent bottom (Costar, Corning, N.Y.), using a SpectraMAX 250 microplate reader from Molecular Devices (Sunnyvale, Calif.). The data were analyzed by finding the maximum slope (in milli-absorbance units per minute) of absorbance measurements made during the interval while between 10 and 40% of the substrate was oxidized. Results obtained with this assay are illustrated in FIGS. 1 and 5.

The mean half-life in sera of mice injected for the first time with PKS uricase coupled to six strands of 10-kDa PEG per subunit (6x10-kDa PEG PKS) was 29±14 hours, based on data from sera obtained 24 and 72 hours after the injection. In separate experiments, it was established that the detectable uricolytic activity in the sera of mice injected with PEG-uricase ceclines during storage at −20° C. and that maximal recovery of this activity is obtained by a 4-hour incubation at 37° C. prior to assay. FIG. 5 shows that the recovery of uricolytic activity after repeated weekly injections of 6x10-kDa PEG PKS uricase was greatest when the enzyme was purified by Mono Q column chromatography, as in Example 1, prior to PEGylation according to the method of Example 3. Recovery was highest after the injection of conjugates prepared from the high-salt eluate pool of Example 1 (see FIG. 1), which has the smallest content of the very large aggregates (see FIG. 2). The same order of relative activities recovered in sera after repeated injections (high salt pool>low salt pool>unfractionated uricase) was observed regardless of whether the UV assay described above or a calorimetric assay adapted from P. Fossati et al. (J Clin Chem (1980) 26:227-231), was used and regardless of whether the sera were incubated at 37° C. before they were assayed.

Example 6

Enzyme-Linked Immunosorbent Assay (ELISA) of Sera from Mice Injected with PEG-Uricase

Non-competitive ELISA analyses were performed with porcine uricase bound to 96-well Immulon 2 plates (Dynex
The primary antisera were from mice injected with uricase or 6x10-kDa PEG conjugates prepared according to the method of Example 3. The secondary antibody was goat anti-mouse IgG coupled to horseradish peroxidase (Calbiochem-Novabiochem #401253, La Jolla, Calif.) and the substrate was o-phenylenediamine dihydrochloride (Sigma P-9187, St. Louis, Mo.), as described by B. Porstmann et al. (J. Clin. Chem. Clin. Biochem. (1981) 19:435-440).

FIG. 6 illustrates the results of the non-competitive ELISA analyses. The results demonstrate that the 6x10-kDa PEG PKS uricase synthesized according to the method of Example 3 from the high-salt eluate from the Mono Q column of Example 1 did not produce detectable immune responses in any of the eight mice that received weekly injections for six weeks. A few mice injected with conjugates prepared from unfractionated PKS uricase according to the method of Example 3 showed low but detectable immune responses. The highest incidence of immune responses was in mice injected with conjugates prepared according to the method of Example 3 from the low-salt eluate pool from the Mono Q column of Example 1.

Without the benefit of the Light scattering detector for the size-exclusion HPLC analyses, as described in Example 2, it would not have been apparent that the presence of the largest aggregates, not of the octameric form of uricase, is associated with progressively decreased recovery of PEG-uricase conjugates after repeated injections, as observed in Example 5 (FIG. 5) and with an increase in immunogenicity in BALB/c mice, as observed in Example 6 (FIG. 6). These results have important implications for the specifications of the uricase used as a starting material for the production of PEG-uricase for clinical use.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereeto without departing from the spirit and scope of that which is described and claimed.

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What is claimed is:

1. A purified urate oxidase (uricase) wherein greater than 95% of said uricase is in the tetrameric and octameric forms.

2. The uricase of claim 1, wherein said uricase is a mammalian uricase.

3. The uricase of claim 2, wherein said uricase is porcine liver uricase, bovine liver uricase or ovine liver uricase.

4. The uricase of claim 1, wherein said uricase is recombinant.

5. The uricase of claim 4, wherein said uricase has the amino acid sequence of porcine, bovine, ovine or baboon liver uricase.

6. The uricase of claim 4, wherein said uricase is chimeric.

7. The uricase of claim 6, wherein said chimeric uricase contains portions of porcine liver uricase and portions of baboon liver uricase.

8. The uricase of claim 7, wherein said chimeric uricase is porcine uricase in which arginine residue 291 of SEQ ID...
NO:1 has been replaced by lysine (R291K) and threonine residue 301 of SEQ ID NO:1 has been replaced by serine (T301S) (PKS uricase).

9. The uricase of claim 4, wherein said uricase has the amino acid sequence as set forth in SEQ ID NO:2, wherein tyrosine residue 97 has been replaced by histidine (Y97H).

10. The uricase of claim 1, wherein said uricase is a fungal or microbial uricase.

11. The uricase of claim 10, wherein said fungal or microbial uricase is isolated from Aspergillus flavus, Arthrobacter globiformis, Bacillus tetrameric and octameric forms.

12. The uricase of claim 1, wherein said uricase is an invertebrate uricase.

13. The uricase of claim 12, wherein said invertebrate uricase is isolated from Drosophila melanogaster or Drosophila pseudoobscura, or is a recombinant enzyme having the amino acid sequence of any one thereof.

14. The uricase of claim 1, wherein said uricase is a plant uricase.

15. The uricase of claim 14, wherein said plant uricase is isolated from root nodules of Glycine max or is a recombinant enzyme having the amino acid sequence thereof.

16. The uricase of claim 1, wherein greater than 97% of said uricase is in the tetrameric and octameric forms.

17. A purified chimeric urate oxidase (urate oxidase), wherein greater than 95% of said uricase is in the tetrameric and octameric forms and wherein said chimeric uricase is porcine uricase in which arginine residue 291 of SEQ ID NO:1 has been replaced by lysine (R291K) and threonine residue 301 of SEQ ID NO:1 has been replaced by serine (T301S) (PKS uricase).

18. A conjugate comprising a purified urate oxidase (urate oxidase) conjugated to poly(ethylene glycol) or poly(ethylene oxide), wherein greater than 95% of said uricase is in the tetrameric and octameric forms.

19. The conjugate of claim 18, wherein greater than 97% of said uricase is in the tetrameric and octameric forms.

20. The conjugate of claim 18, wherein said poly(ethylene glycol) is monomethoxy poly(ethylene glycol).

21. The conjugate of claim 18, wherein said uricase is conjugated to said poly(ethylene glycol) or poly(ethylene oxide) via a linkage selected from the group consisting of urethane (carbamate), secondary amine and amide.

22. The conjugate of claim 18, wherein said poly(ethylene glycol) or poly(ethylene oxide) has an average molecular weight of about 10 kDa to 60 kDa.

23. The conjugate of claim 22, wherein said poly(ethylene glycol) or poly(ethylene oxide) has an average molecular weight of about 10 kDa to 30 kDa.

24. The conjugate of claim 18, wherein the average number of strands of said poly(ethylene glycol) or poly(ethylene oxide) conjugated to said uricase is between about 2 and 12 per uricase subunit.

25. The conjugate of claim 24, wherein said average number of strands of said poly(ethylene glycol) or poly(ethylene oxide) is between about 6 and 10 per uricase subunit.

26. The conjugate of claim 25, wherein said average number of strands of said poly(ethylene glycol) or poly(ethylene oxide) is between about 7 and 9 per uricase subunit.

27. The conjugate of claim 18, wherein said poly(ethylene glycol) or poly(ethylene oxide) is linear.

28. The conjugate of claim 18, wherein said poly(ethylene glycol) or poly(ethylene oxide) is branched.

29. A purified fragment of urate oxidase (urate oxidase), wherein said fragment is a recombinant uricase that has been truncated at the amino terminus, at the carboxyl terminus, or at both the amino and carboxyl termini, and wherein greater than 95% of said truncated uricase is in the tetrameric and octameric forms.

30. The purified uricase fragment of claim 29, wherein greater than 97% of said truncated uricase is in the tetrameric and octameric forms.

31. A conjugate comprising a purified urate oxidase (urate oxidase) conjugated to poly(ethylene glycol) or poly(ethylene oxide), wherein greater than 95% of said uricase is in the tetrameric and octameric forms and wherein the average number of strands of said poly(ethylene glycol) or poly(ethylene oxide) conjugated to said uricase is between about 2 and about 10 per uricase subunit.

32. The conjugate of claim 31, wherein greater than 97% of said uricase is in the tetrameric and octameric forms.

33. The conjugate of claim 31, wherein said poly(ethylene glycol) or poly(ethylene oxide) has an average molecular weight of about 10 kDa to 60 kDa.

34. The uricase conjugate of claim 33, wherein said poly (ethylene glycol) or poly(ethylene oxide) has an average molecular weight of about 10 kDa to 30 kDa.

35. A conjugate comprising a purified urate oxidase (urate oxidase) conjugated to poly(ethylene glycol) or poly(ethylene oxide), wherein greater than 95% of said uricase is in the tetrameric and octameric forms, wherein the average number of strands of said poly(ethylene glycol) or poly(ethylene oxide) conjugated to said uricase is between about 2 and 10 per uricase subunit and wherein said poly(ethylene glycol) or poly(ethylene oxide) has an average molecular weight of about 10 kDa to 60 kDa.

36. The uricase conjugate of claim 35, wherein greater than 97% of said uricase is in the tetrameric and octameric forms.

37. A pharmaceutical composition for lowering uric acid levels in a body fluid or tissue, comprising a purified urate oxidase (urate oxidase) conjugated to poly(ethylene glycol) or poly (ethylene oxide) and a pharmaceutically acceptable carrier, wherein greater than 95% of said uricase is in the tetrameric and octameric forms.

38. The pharmaceutical composition of claim 37, wherein said composition is stabilized by lyophilization and dissolves upon reconstitution to provide a solution suitable for parenteral administration.

39. An isolated urate oxidase (urate oxidase) prepared by a method comprising separating uricase aggregates larger than uricase tetramers and octamers and excluding such aggregates from the isolated uricase, wherein greater than 95% of said isolated uricase is in the tetrameric and octameric forms.

40. The uricase of claim 39, wherein greater than 97% of said isolated uricase is in the tetrameric and octameric forms.