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(54) **METHOD OF DIAGNOSING ALZHEIMER'S DISEASE USING SERUM GLYCOPROTEIN AS BIOMARKER**

VERFAHREN ZUR DIAGNOSE VON MORBUS ALZHEIMER UNTER VERWENDUNG VON SERUMGLYKOPROTEIN ALS BIOMARKER

PROCÉDÉ DE DIAGNOSTIC DE LA MALADIE D'ALZHEIMER EN UTILISANT UNE GLYCOPROTÉINE DE SÉRUM COMME MARQUEUR BIOLOGIQUE

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(56) References cited:

- **JOHNSON G ET AL: "CEREBROSPINAL FLUID PROTEIN VARIATIONS IN COMMON TO ALZHEIMER'S DISEASE AND SCHIZOPHRENIA" APPLIED AND THEORETICAL ELECTROPHORESIS, MACMILLAN PRESS LTD, US, vol. 3, no. 2, 1 January 1992 (1992-01-01), pages 47-53, XP009014286**
- **YASUKAWA ZENTA ET AL: "Inflammation-dependent changes in alpha 2,3-, alpha 2,6-, and alpha 2,8-sialic acid glycotopes on serum glycoproteins in mice" [Online] September 2005 (2005-09), GLYCOBIOLOGY, VOL. 15, NR. 9, PAGE(S) 827-837, XP002485315 ISSN: 1460-2423 Retrieved from the Internet: URL: <http://glycob.oxfordjournals.org/cgi/content/abstract/15/9/827> [retrieved on 2008-06-24] ISSN Online: 1460-2423 ISSN Print: 0959-6658 pages 828-830**
- **AISEN P S ET AL: "INFLAMMATORY MECHANISMS IN ALZHEIMER'S DISEASE: IMPLICATIONS FOR THERAPY" AMERICAN JOURNAL OF PSYCHIATRY, AMERICAN PSYCHIATRIC ASSOCIATION, WASHINGTON, DC, US, vol. 151, no. 8, 1 August 1994 (1994-08-01), pages 1105-1113, XP002070242 ISSN: 0002-953X**

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**EP 1 901 066 B1**

- KITAZUME ET AL.: "Alzheimer's beta-secretase, beta-site amyloid precursor protein-cleaving enzyme, is responsible for cleavage secretion of a Golgi-resident sialyltransferase"  
PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC, vol. 98, no. 24, 20 November 2001 (2001-11-20), pages 13554-13559, XP002224671 ISSN: 0027-8424
- JOHNSON ET AL.: 'Cerebrospinal fluid protein variations in common to Alzheimer's disease and schizophrenia' APPLIED AND THEORETICAL ELECTROPHORESIS vol. 3, 1992, pages 47 - 53, XP009014286
- SPAGNUOLO ET AL.: 'The Binding of Haptoglobin to Apolipoprotein A1: Influence of Hemoglobin and Concanavalin A' BIOL. CHEM. vol. 384, 2003, pages 1593 - 1596, XP009062164
- KITAZUME ET AL.: 'Alzheimer's beta-secretase cleaves a glycosyltransferase as a physiological substrate' GLYCOCONJUGATE JOURNAL vol. 20, 2004, pages 59 - 62, XP003003351
- KITAZUME ET AL.: 'In Vivo Cleavage of alpha2,6-Sialyltransferase by Alzheimer beta-secretase' THE JOURNAL OF BIOLOGICAL CHEMISTRY vol. 280, no. 9, March 2005, pages 8589 - 8595, XP003003352

## Description

### TECHNICAL FIELD

**[0001]** The present invention relates to the use of an agent and a method for diagnosing Alzheimer's disease. More specifically, the present invention relates to the use of an agent and a method for diagnosing Alzheimer's disease, in which an  $\alpha$  2,6-sialyl residue-containing glycoprotein is used as a marker.

### BACKGROUND ART

**[0002]** Alzheimer's disease is a disease attended with a wide range of symptoms of neurodegenerative dementia. Generation of amyloid  $\beta$  peptides ( $A\beta$ ) is considered to be a cause of Alzheimer's disease (Selkoe, D. J. (2001) *Physiol. Rev.* 81, 741-766; and Iwata, N. et al., (2001) *Science* 292, 1550-1552). An amyloid precursor protein (APP) is cleaved with  $\beta$ -secretase, and as a result, a soluble  $NH_2$ -terminal fragment ( $APPs\beta$ ) and a 12-kDa COOH-terminal fragment (C99) are generated. The latter fragment remains bound to a membrane. C99 is further cleaved with  $\gamma$ -secretase, and then pathogenic  $A\beta$  is generated (De Strooper, B. et al., (1998) *Nature (London)* 391, 387-390; and Wolfe, M. S. et al., (1990) *Nature (London)* 398, 513-517.) Via another pathway, APP is cleaved with  $\alpha$ -secretase in an  $A\beta$  sequence, and as a result, a soluble  $NH_2$ -terminal fragment ( $APPs\alpha$ ) and a 10-kDa membrane-bound COOH-terminal fragment (C83) are generated (Buxbaum, J. D. et al., (1998) *J. Biol. Chem.* 273, 27765-27767; and Lammich, S. et al., (1999) *Proc. Natl. Acad. Sci. USA* 96, 3922-3927).

**[0003]** It has been reported that there are a large number of markers showing a correlation with Alzheimer's disease. The relationship of a majority of such markers with the pathological changes is unknown, and the diagnostic value has not been necessarily established. Markers that are directly associated with such pathological changes include a decrease in one type of amyloid  $\beta$  peptide ( $A\beta$  1-42) consisting of 42 amino acids contained in cerebrospinal fluid. However, since such  $A\beta$  peptide is easily aggregated to create a precipitate, it becomes a cause of diseases. Accordingly, the amount of free  $A\beta$  is very small, and thus it is theoretically difficult to use such a trace amount of  $A\beta$  as a diagnostic marker. As a matter of fact, it has been known that the amount of such  $A\beta$  peptide is fluctuated only after the symptoms of Alzheimer's disease become severe. Thus, such  $A\beta$  peptide cannot be used as an early diagnostic marker. On the other hand, an increase of the phosphorylated tau protein in cerebrospinal fluid is observed even at an early stage of the disease, and thus it is considered the most excellent biomarker. However, when such phosphorylated tau is increased, neuron death has already progressed. Accordingly, even if a treatment is initiated at such stage, a complete recovery of nerve function cannot be expected. In addition, when such markers are used

in measurements using cerebrospinal fluid (lumbar puncture fluid). In order to collect such material, a special technique is necessary. Since it puts a burden upon patients, a method using the aforementioned markers cannot become a mass screening method.

**[0004]** The present inventors have found that  $\beta$ -secretase cleaves not only an amyloid precursor protein, but also  $\alpha$  2,6-sialyltransferase. Based on this discovery, the inventors have measured the cleaved free  $\alpha$  2,6-sialyltransferase. Thereafter, regarding the fact that such  $\alpha$  2,6-sialyltransferase can be used in diagnoses as a marker for an increase in  $\beta$ -secretase activity, the inventors have previously filed a patent application (Japanese Patent Application No. 2003-382374, published as WO 2005/047897). In the aforementioned patent application, an antibody that specifically recognizes the cleavage site of human soluble  $\alpha$  2,6-sialyltransferase is used. Use of this antibody has enabled quantification of soluble  $\alpha$  2,6-sialyltransferase in human blood or cerebrospinal fluid. Thus, a method for monitoring  $\beta$ -secretase activity has been provided. However, in general, only a trace amount of glycosyltransferase is contained in body fluid, and thus the detection efficiency is not necessarily high. Further, a cleavage site-recognizing antibody has been required for detection of such glycosyltransferase.

**[0005]** The document Johnson et al. (*Applied and Theoretical Electrophoresis*, Vol.3, No. 2, pages 47-53 (1992)) discloses a study showing that haptoglobin is increased in Alzheimer patients.

**[0006]** The document Yasukawa et al. (*Glycobiology*, Vol. 15, No. 9, pages 827-837 (2005)) discloses that that in sera of inflamed mice  $\alpha$  2,6-sialyl glycotopes on glycoproteins are increased. The document further discloses the use of lectin for the detection of the  $\alpha$  2,6-sialyl residue-containing glycoproteins.

**[0007]** Further, from this document the skilled person also learns that a given haptoglobin molecule may comprise  $\alpha$  2,6-sialyl residues or may not.

**[0008]** None of the prior art document discloses a correlation of the presence of  $\alpha$  2,6-sialyl residues on glycoproteins as haptoglobin with Alzheimer's disease

### DISCLOSURE OF THE INVENTION

#### Problems to be Solved by the Invention

**[0009]** In sporadic Alzheimer's disease, which accounts for 95% or more of all types of Alzheimer's diseases, Alzheimer's disease  $\beta$ -secretase activity is increased, and the cleavage of an amyloid precursor protein with this enzyme increasingly takes place. As a result, generation of an amyloid  $\beta$  peptide ( $A\beta$ ) that is a final product is increased, and it is then precipitated in the brain. This precipitate is called senile plaque, which is considered to be a pathologic change in the early stage of Alzheimer's disease. That is to say, an increase in the activity of  $\beta$ -secretase triggers sporadic Alzheimer's disease. If changes in such  $\beta$ -secretase activity can be de-

tected, the aforementioned disease can be diagnosed before the development thereof, and thus it becomes possible to effectively prevent such disease. However, to date, such a method for easily detecting such activity increase with high sensitivity has not previously existed.

**[0010]** It is an object of the present invention to detect a change in the metabolism of a glycoprotein having an  $\alpha$  2,6-sialyl residue, which is contained in blood, so as to provide an agent and a method for diagnosing sporadic Alzheimer's disease.

#### Means for Solving the Problems

**[0011]** As a result of intensive studies directed towards achieving the aforementioned object, the present inventors have found that  $\beta$  -secretase cleaves not only an amyloid precursor protein, but also  $\alpha$  2,6-sialyltransferase, and that the metabolism of a glycoprotein having an  $\alpha$  2,6-sialyl residue that is an enzyme product thereof is changed. Thereafter, the inventors have used lectins specifically binding to  $\alpha$  2,6-sialyl residues (SNA, SSA, and TJA lectins, etc.) to examine changes in such glycoproteins contained in the blood of a patient suffering from Alzheimer's disease. As a result, the inventors have revealed that the quantities of several glycoproteins had been changed (refer to Example 1). This result demonstrated that an  $\alpha$  2,6-sialyl residue-containing glycoprotein can be used as a marker in the diagnosis of Alzheimer's disease.

**[0012]** The present invention provides:

(1) *In vitro* use of an agent for diagnosing Alzheimer's disease, said agent comprising lectin used for detecting an  $\alpha$  2,6-sialyl residue-containing glycoprotein, wherein said lectin specifically binds to  $\alpha$  2,6-sialyl residues of an  $\alpha$  2,6-sialyl residue-containing glycoprotein.

(2) The use according to (1), wherein the  $\alpha$  2,6-sialyl residue-containing glycoprotein is a 180-kD glycoprotein, a 95-kD glycoprotein, or a 40-kD glycoprotein.

(3) The use according to (1), wherein said agent further comprises an anti-haptoglobin antibody used for detecting a 40-kD glycoprotein that contains  $\alpha$  2,6-sialyl residue.

(4) A kit for diagnosing Alzheimer's disease, which comprises lectin and an anti-haptoglobin antibody, wherein said lectin specifically binds to  $\alpha$  2,6-sialyl residues of an  $\alpha$  2,6-sialyl residue-containing glycoprotein.

(5) A method for diagnosing Alzheimer's disease, which comprises detecting or measuring an  $\alpha$  2,6-sialyl residue-containing glycoprotein contained in a human-derived sample, using a lectin that specifi-

cally binds said  $\alpha$  2,6-sialyl residue.

(6) The method according to (5), wherein the  $\alpha$  2,6-sialyl residue-containing glycoprotein is a 180-kD glycoprotein, a 95-kD glycoprotein, or a 40-kD glycoprotein.

(7) the method according to (5) or (6), wherein a 40-kD glycoprotein that contains  $\alpha$  2,6-sialyl residue is detected or measured by using an anti-haptoglobin antibody.

(8) The method according to (5), wherein a 40-kD glycoprotein that contains  $\alpha$  2,6-sialyl residue is detected or measured by sandwich assay using lectin and an anti-haptoglobin antibody, wherein said lectin specifically binds to  $\alpha$  2,6-sialyl residues of an  $\alpha$  2,6-sialyl residue-containing glycoprotein.

(9) The method, according to any of (5) to (8), wherein the human-derived sample is a blood sample.

#### BEST MODE FOR CARRYING OUT THE INVENTION.

**[0013]** The embodiments of the present invention will be described in detail below.

**[0014]** The use and method for diagnosing Alzheimer's disease of the present invention is characterized in that an  $\alpha$  2,6-sialyl residue-containing glycoprotein is used as a marker. More specifically, a 180-kD glycoprotein, a 95-kD glycoprotein, or a 40-kD glycoprotein, which contains sialic acid, can be used as a marker. All of such glycoproteins can be detected using lectin. In addition, the 40-kD glycoprotein can be detected using an anti-haptoglobin antibody.

**[0015]** As an example of the diagnostic method of the present invention, the amount of an  $\alpha$  2,6-sialyl residue-containing glycoprotein existing in a subject-derived sample is compared with the amount of an  $\alpha$  2,6-sialyl residue-containing glycoprotein existing in a sample derived from a control subject, so as to diagnose Alzheimer's disease.

**[0016]** Such an  $\alpha$  2,6-sialyl residue-containing glycoprotein (preferably, a 180-kD glycoprotein, a 95-kD glycoprotein, or a 40-kD glycoprotein) can be detected or measured by an assay using lectin or an antibody that specifically recognizes the  $\alpha$  2,6-sialyl residue-containing glycoprotein (for example, an anti-haptoglobin antibody that can be used for the 40-kD glycoprotein).

**[0017]** That is, the binding of an  $\alpha$  2,6-sialyl residue-containing glycoprotein with lectin or the aforementioned antibody can be analyzed by binding a labeling substance used for enzyme labeling, color labeling, radiolabeling, or luminescent labeling, etc., to lectin, the aforementioned antibody, or a secondary antibody, and then detecting or measuring such a labeling substance. Examples of an immunoassay that can be carried out in the present invention include ELISA, Western blotting, im-

munoprecipitation, slot or dot blot assay, immunohistological staining, radio immuno assay (RIA), fluoroimmunoassay, and an immunoassay using an avidin-biotin or streptoavidin-biotin system, but examples are not limited thereto.

**[0018]** A human-derived sample can be used in the diagnostic method of the present invention. The type of such a human-derived sample is not particularly limited, as long as an a 2,6-sialyl residue-containing glycoprotein (preferably, a 180-kD glycoprotein, a 95-kD glycoprotein, or a 40-kD glycoprotein) can be detected or measured in the sample. Examples of such a human-derived sample used herein include blood (serum, plasma, etc.), urine, saliva, and a brain-derived sample. A preferred example is blood (serum or plasma).

**[0019]** The type of lectin used in the present invention is not particularly limited, as long as it recognizes such an a 2,6-sialyl residue-containing glycoprotein and detects it. Examples of such lectin used herein include SNA lectin (*Sambucus nigra* lectin), SSA lectin (*Sambucus sieboldiana* lectin), and TJA lectin (*Trichosanthes japonica* lectin). The lectin used in the present invention may be labeled, as necessary. As the labeling substance, a labeling substance for a labeled antibody as described later in the present specification can be used.

**[0020]** An example of the antibody used in the present invention is an anti-haptoglobin antibody. It has been known that haptoglobin is fluctuated when acute inflammation or hemolytic anemia occurs. Accordingly, it is necessary to make the differential diagnosis between the aforementioned diseases and Alzheimer's disease. Acute inflammation can be differentiated by a common biochemical examination of blood such as the use of a C reactive protein value. Hemolytic anemia can be differentiated by a common examination used for icterus, hyperbilirubinemia. In the case of hemolytic anemia, advanced examinations such as the measurement of erythrocyte life span or the measurement of iron metabolism cycle may be necessary in some cases. However, such advanced examinations are carried out only in extremely exceptional cases. That is, Alzheimer's disease can be easily differentiated from the aforementioned diseases by a common biochemical examination with blood specimen, and thus the use of an anti-haptoglobin antibody does not become an impediment to the diagnosis of Alzheimer's disease.

**[0021]** Moreover, in the case of a 180-kD glycoprotein and a 95-kD glycoprotein, such a glycoprotein is purified, and thereafter, an animal is immunized with the glycoprotein or a partial peptide thereof used as an antigen, so as to obtain an antibody. The antibody used in the present invention can be either a polyclonal antibody or a monoclonal antibody. Such an antibody can be produced by common methods.

**[0022]** In the case of a polyclonal antibody, for example, a mammal is immunized with the aforementioned protein or a partial peptide thereof used as an antigen, and blood is then collected from the mammal. Thereafter,

an antibody can be separated and purified from the collected blood. For example, mammals such as a mouse, a hamster, a guinea pig, a chicken, a rat, a rabbit, a dog, a goat, a sheep, or a bovine can be immunized. As an immunization method, a common immunization method known to persons skilled in the art may be used, and an antigen may be administered one or more times, for example.

**[0023]** Such an antigen can be administered two or three times at intervals of 7 to 30 days, and particularly 12 to 16 days. As a single dose, approximately 0.05 to 2 mg of antigen can be administered, for example. Also, an administration route is not particularly limited. Subcutaneous administration, intracutaneous administration, intraperitoneal administration, intravenous administration, intramuscular administration can be selected, as appropriate. It is preferable to administer an antigen via an intravenous, intraperitoneal, or subcutaneous administration route. Furthermore, such an antigen can be dissolved in a suitable buffer that contains a commonly used adjuvant such as a complete Freund's adjuvant, RAS [MPL (Monophosphoryl Lipid A) + TDM (Synthetic Trehalose Dicorynomycolate) + CWS (Cell Wall Skeleton) adjuvant system], or aluminum hydroxide, before use. However, there may also be cases where the aforementioned adjuvants cannot be used depending on an administration route, other administration conditions, etc. The term "adjuvant" is used herein to mean a substance, which is administered together with an antigen and which non-specifically enhances an immune reaction to the antigen.

**[0024]** An immunized mammal is raised for 0.5 to 4 months. Thereafter, a small amount of serum is collected as a sample from the ear vein of the mammal, and an antibody titer thereof can be then measured. If such an antibody titer is increased, an antigen is administered for a suitable number of times depending on the situation. A booster can be carried out with 10 to 1,000 µg of antigen, for example. One to two months after the final administration, blood is collected from the immunized mammal according to an ordinary method, and it is then treated at 56°C for 30 minutes to inactivate a complement system. Thereafter, a specific antibody is purified by affinity chromatography. As an affinity carrier, an antigen peptide that has been immobilized on Affigel can be used, for example. The aforementioned blood is separated and purified by an ordinary method such as centrifugation, precipitation using ammonium sulfate or polyethylene glycol, or chromatography such as gel filtration chromatography, ion exchange chromatography or affinity chromatography, so as to obtain a polyclonal antibody in the form of a polyclonal antiserum. It is to be noted that such serum may be treated at 56°C for 30 minutes to inactivate a complement system.

**[0025]** A monoclonal antibody can be produced using a hybridoma obtained by the cell fusion between an antibody-generating cell and a myeloma cell line. A hybridoma that generates a monoclonal antibody can be ob-

tained by the following cell fusion method.

**[0026]** As antibody-generating cells, splenic cells, lymph node cells, B lymphocytes, or other types of cells, obtained from the immunized animal, can be used. As an antigen, the same protein or peptide as in the case of a polyclonal antibody can be used. As an animal to be immunized, a mouse, a rat can be used. An antigen is administered to such animals according to common methods. For example, a suspension or an emulsion is prepared by mixing an adjuvant such as a complete Freund's adjuvant or an incomplete Freund adjuvant with an antigen peptide. Thereafter, the thus prepared suspension or emulsion is administered intravenously, subcutaneously, intracutaneously, intraperitoneally, etc., to an animal to be immunized several times, so that the animal can be immunized. Thereafter, antibody-generating cells such as splenic cells are obtained from the thus immunized animal, and the obtained splenic cells are then fused with myeloma cells according to a known method (G. Kohler et al., Nature, 256 495 (1975)), so as to produce hybridomas.

**[0027]** Examples of a myeloma cell line used in cell fusion include P3X63Ag8, P3U1 line, and Sp2/0 line, in the case of mice. When cell fusion is carried out, a fusion promoter such as polyethylene glycol or Sendai virus is used. In order to select hybridomas after completion of the cell fusion, a hypoxanthine-aminopterin-thymidine (HAT) medium can be used according to common methods. Hybridomas obtained as a result of the cell fusion can be cloned by the limiting dilution method or the like. Further, the obtained cells are screened by enzyme immunoassay, so as to obtain a cell line that generates a monoclonal antibody capable of specifically recognizing an  $\alpha$  2,6-sialyl residue-containing glycoprotein.

**[0028]** In order to produce a monoclonal antibody of interest from the thus obtained hybridomas, the hybridomas may be cultured by a common cell culture method or an ascites formation method, and the monoclonal antibody may be purified from the culture supernatant or ascites. Such monoclonal antibody can be purified from such culture supernatant or ascites according to common methods. For example, methods such as ammonium sulfate fractionation, gel filtration, ion exchange chromatography, and affinity chromatography can be used in combination, as appropriate.

**[0029]** The antibody used in the present invention can also be used as a labeled antibody.

**[0030]** By producing such a labeled antibody, an  $\alpha$  2,6-sialyl residue-containing glycoprotein can be easily detected or measured. In addition, the antibody used in the present invention, or a secondary antibody that binds to an  $\alpha$  2,6-sialyl residue-containing glycoprotein as an antigen thereof, can also be labeled before use. The type of labeling substance for the antibody of the present invention or the secondary antibody thereof, and a labeling method thereof, can be appropriately selected from those known to persons skilled in the art.

**[0031]** When an enzyme is used as a labeling sub-

stance, horseradish peroxidase, alkaline phosphatase, glucose oxidase,  $\beta$ -galactosidase, glucoamylase, carbonic anhydrase, acetylcholine esterase, lysozyme, malate dehydrogenase, glucose-6-phosphate dehydrogenase, can be used as such a labeling substance. Examples of a method of labeling the antibody of the present invention, the secondary antibody thereof, or the fragment thereof (F(ab')<sub>2</sub> fragment, Fab' fragment, etc.) with such enzyme include: a method comprising oxidizing a sugar chain of enzyme with periodic acid and allowing the amino acid of the aforementioned antibody to bind to the generated aldehyde group; and a method of introducing a maleimide group, a pyridyl sulfide group into the enzyme and allowing such a group to bind to a thiol group existing in the Fab' fragment of the aforementioned antibody.

**[0032]** When an enzyme is used as a labeling substance, a test sample and a labeled antibody are subjected to incubation. Thereafter, a free labeled antibody is eliminated by washing, and the substrate of the aforementioned labeling enzyme is then allowed to act thereon. The reaction is measured based on color development, so that the labeled antibody can be detected. When an antibody is labeled with peroxidase for example, hydrogen peroxide used as a substrate is combined with diaminobenzidine or O-phenylenediamine used as a coloring agent, so as to generate a brown or yellow color. When an antibody is labeled with glucoseoxidase, 2,2'-azino-di-(3-ethylbenzothiazolin-6-sulfonate) (ABTS) can be used as a substrate, for example.

**[0033]** When a fluorochrome is used as a labeling substance, the antibody used in the present invention or a secondary antibody thereof can be labeled with a fluorochrome such as FITC (fluorescein isothiocyanate) or TRITC (tetramethylrhodamine B isothiocyanate). The antibody used in the present invention or a secondary antibody thereof can be bound to a fluorochrome by a common method.

**[0034]** When a coloring substance is used as a labeling substance, colloidal metal or color latex can be used as a labeling substance, for example. Representative examples of colloidal metal include colloidal metal particles that are the disperse particles of gold sol, silver sol, selenium sol, tellurium sol, platinum sol, etc. With regard to the size of such a colloidal metal particle, the diameter thereof is generally between approximately 3 and 60 nm. Representative examples of color latex include synthetic latexes such as polystyrene latex colored with a pigment of red, blue, or other colors. As such latex, natural latex such as natural rubber latex can be used. With regard to the size of such color latex, the diameter thereof can be selected from the size ranging from approximately several tens of to several hundreds of nm. Commercially available products can be directly used as such coloring substances. However, there may also be cases where such a commercially available product is further processed, or where a coloring substance can also be produced by a known method.

**[0035]** The antibody of the present invention or a secondary antibody thereof can be bound to a coloring substance by a common method. When such a coloring substance is a gold colloidal particle that is the disperse particle of gold sol, for example, it is generally possible that the aforementioned antibody will be physically bound to the gold sol by mixing them at room temperature.

**[0036]** As a labeling substance, affinity labeling substances (e.g. biotin, etc.) or isotopic labeling substances (e.g.  $^{125}\text{I}$ , etc.) can be used, as well as the aforementioned substances.

**[0037]** In the method of the present invention, an  $\alpha$  2,6-sialyl residue-containing glycoprotein can be detected or measured by ELISA, Western blotting, immunoprecipitation, slot or dot blot assay, immunohistological staining, radio immune assay (RIA), " fluoroimmunoassay, an immunoassay using an avidin-biotin or streptoavidin-biotin system, etc. These methods are well known to persons skilled in the art.

**[0038]** When Western blotting is applied, for example, a glycoprotein can be detected by the method described in Example 2 of the present specification.

**[0039]** In addition, when an antibody such as anti-haptoglobin can be used, quantification can be carried out by the following sandwich lectin ELISA method or a common sandwich ELISA method. A plate used in ELISA has previously been coated with an anti-haptoglobin antibody. Thereafter, haptoglobin contained in a sample is trapped on the plate. According to the sandwich lectin ELISA method, the trapped haptoglobin is allowed to react with biotinylated lectin (SNA, SSA, TJA, etc.). For detection, the resultant is allowed to react with streptavidin-horseradish peroxidase (HRP). On the other hand, according to a common sandwich ELISA method, an antibody, which differs from the antibody that has been applied onto a plate, is used for detection. In this case, an antibody used for detection (or a Fab fragment thereof) that is directly labeled with horseradish peroxidase can be used in the reaction. (Otherwise, a secondary antibody labeled with horseradish peroxidase can be used.) In both types of ELISA methods, a coloring substrate is added to develop a color, and the concentration of the color is then measured using a spectrophotometer. A calibration curve has previously been produced with a standard sample containing a known concentration of haptoglobin. Using such a calibration curve, a quantitative value can be calculated.

**[0040]** The present invention also provides a diagnostic kit for diagnosing Alzheimer's disease. Such a diagnostic kit comprises: (1) lectin for detecting an  $\alpha$  2,6-sialyl residue-containing glycoprotein; and (2) an antibody against a 180-kD glycoprotein, 95-kD glycoprotein or 40-kD glycoprotein that contains  $\alpha$  2,6-sialyl residue (e.g. an anti-haptoglobin antibody for detecting a 40-kD glycoprotein, etc.). The diagnostic kit may further comprise a secondary antibody to which a labeling substance capable of generating signals has bound, as well as the aforementioned components. Examples of a secondary

antibody used herein include an antibody capable of binding to the aforementioned primary antibody and an antibody capable of recognizing an  $\alpha$  2,6-sialyl residue-containing glycoprotein and binding thereto (however, which recognizes a site differing from the binding site of the primary antibody and binds thereto). When an antibody capable of recognizing an  $\alpha$  2,6-sialyl residue-containing glycoprotein and binding thereto is used as a secondary antibody, sandwich immunoassay (e.g. sandwich ELISA, etc.) can be carried out.

**[0041]** In the diagnostic kit of the present invention, the primary antibody of the present invention may have previously been solid-phased, or it may have previously been labeled with a labeling substance. The type of a solid phase used in the diagnostic kit of the present invention is not particularly limited. Examples of such a solid phase include polymers such as polystyrene, and insoluble carriers such as glass beads, magnetic particles, a microplate, a filter used in immunochromatography, or a glass filter.

**[0042]** The diagnostic kit of the present invention may further comprise other types of any given components. Examples of such other types of any given components include an enzyme used in labeling, a substrate thereof, a radioisotope, a luminescent substance, a fluorescent substance, a coloring substance, a buffer, and a plate, but examples are not limited thereto.

**[0043]** Also, the form of the diagnostic kit of the present invention is not particularly limited. For the purpose of quickly and simply making diagnoses, the diagnostic kit of the present invention can be provided in the form of an integral-type diagnostic kit, wherein constituents for the diagnostic kit of the present invention are integrated. The form of such an integral-type diagnostic kit is not particularly limited. Examples of the form of such an integral-type diagnostic kit include a cassette-type kit used in immunochromatography and a cartridge-type kit used in competitive immunoassay.

**[0044]** The present invention will be more specifically described in the following examples. EXAMPLES

Example 1: Detection of  $\alpha$  2,6-sialyl residue-containing glycoprotein by lectin-blotting method

**[0045]** A human serum or plasma sample (corresponding to 20  $\mu\text{g}$  of protein) was mixed with a Laemmli sample buffer, and the mixture was then heated. Thereafter, the mixture was subjected to SDS/PAGE using 4/20% gradient polyacrylamide gel. Electrophoresis was performed at a constant current of 40 mA for 55 minutes. The separated protein was electrically transcribed onto a PVDF membrane at a constant current of 260 mA for 50 minutes. Thereafter, the membrane was blocked in 1% bovine serum albumin (BSA)-phosphate buffered saline (PBS) for one or more hours. Thereafter, biotinylated lectin (SSA) diluted with 1% BSA-PBS was allowed to react with the PVDF membrane for 1 hour. (In this experiment, biotin-SSA (#300442; Seikagaku Corp.) was used in a

concentration of 1  $\mu\text{g/ml}$ .) The membrane was washed three times with 0.05% Tween 20 (surfactant) in PBS for 15 minutes for each time. Thereafter, the resultant was allowed to react with streptavidin-horseradish peroxidase conjugate (HRP) (#RPN 1231V; Amersham) diluted with 1% BSA-PBS for 1 hour. Thereafter, the membrane was washed again with a washing solution three times for 15 minutes for each time. A chemoluminescent substrate (PierceSuperSignal West DuraExtended Duration Substrate) was used, and the band of an  $\alpha$  2,6-sialyl residue-containing glycoprotein was detected by a lumino-image analyzer (LAS-1000plus; Fuji Film, Tokyo), followed by quantification. The results are shown in Figure 1. The results demonstrated that a 180-kD glycoprotein, a 95-kD glycoprotein, and a 40-kD glycoprotein, which contain  $\alpha$  2,6-sialyl residue, were increased in patients suffering from Alzheimer's disease.

#### Example 2: Western blotting method

**[0046]** Taking into consideration the fact that such glycoproteins contain  $\alpha$  2,6-sialyl residue and the molecular weights thereof, an attempt was made to identify a 180-kD glycoprotein, a 95-kD glycoprotein, and a 40-kD glycoprotein. An antibody reacting with a human serum glycoprotein used as a candidate was purchased and examined. As a result, it was found that the 40-kD glycoprotein is haptoglobin. A detection method thereof will be described below.

**[0047]** A human serum or plasma sample (corresponding to 0.04  $\mu\text{g}$  of protein) was mixed with a Laemmli sample buffer, and the mixture was then heated. Thereafter, the mixture was subjected to SDS/PAGE using 4/20% gradient polyacrylamide gel. Electrophoresis was performed at a constant current of 40 mA for 55 minutes. The separated protein was electrically transcribed onto a PVDF membrane at a constant current of 260 mA for 50 minutes. Thereafter, the membrane was blocked in 5% skimmed milk-0.1% Tween 20 in Tris buffered saline (TBS) for one or more hours. Subsequently, a rabbit anti-human haptoglobin antibody (Sigma H-8636) (12  $\mu\text{g/ml}$ ) diluted with 3% skimmed milk-TBS at a ratio of 1 : 1,000 was allowed to react with a nitrocellulose membrane for 1 hour. Thereafter, the membrane was washed three times with 0.1% Tween 20 (surfactant) in TBS for 15 minutes for each time. Thereafter, the resultant was allowed to react with anti-rabbit IgG, horseradish peroxidase (#NA934V; Amersham) (1  $\mu\text{g/ml}$ ) diluted with 3% skimmed milk-TBS at a ratio of 1 : 1,000 for 1 hour. Thereafter, the membrane was washed again with a washing solution three times for 15 minutes for each time. A chemoluminescent substrate (PierceSuperSignal West DuraExtended Duration Substrate) was used, and the band of an  $\alpha$  2,6-sialyl residue-containing glycoprotein was detected by a lumino-image analyzer (LAS-1000plus; Fuji Film, Tokyo), followed by quantification. The results of the Western blotting using an anti-haptoglobin antibody are shown in Figure 2. An increase in haptoglobin was

confirmed in Alzheimer's disease. A calibration curve has previously been produced with a standard sample containing normal human serum or a known concentration of haptoglobin (#H0138; Sigma). Using such a calibration curve, a quantitative value can be calculated.

#### BRIEF DESCRIPTION OF THE DRAWINGS

##### [0048]

Figure 1 shows human serum that was stained with SSA lectin.

Figure 2 shows detection of a 40kD-glycoprotein with a haptoglobin antibody.

#### INDUSTRIAL APPLICABILITY

**[0049]** In the present invention, an  $\alpha$  2,6-sialyl residue-containing glycoprotein, which exists in a large amount in blood, is used as a marker. Thus, it is possible to make diagnoses using only a trace amount of blood sample. When compared with the conventional diagnostic methods using cerebrospinal fluid or a large amount of blood, a lesser burden is imposed upon a subject. In addition, in the present invention, a special antibody is not required, and lectin that can be prepared in a large amount in an inexpensive way or antibodies that can be easily produced (e.g. anti-haptoglobin antibody, etc.) are used. Accordingly, a method for screening many analytes with high sensitivity can be easily established.

#### Claims

1. *In vitro* use of an agent for diagnosing Alzheimer's disease, said agent comprising lectin used for detecting an  $\alpha$  2,6-sialyl residue-containing glycoprotein, wherein said lectin specifically binds to  $\alpha$  2,6-sialyl residues of an  $\alpha$  2,6-sialyl residue-containing glycoprotein.
2. The use according to claim 1, wherein the  $\alpha$  2,6-sialyl residue-containing glycoprotein is a 180-kD glycoprotein, a 95-kD glycoprotein, or a 40-kD glycoprotein.
3. The use according to claim 1, wherein said agent further comprises an anti-haptoglobin antibody used for detecting a 40-kD glycoprotein that contains  $\alpha$  2,6-sialyl residue.
4. A kit for diagnosing Alzheimer's disease, which comprises lectin and an anti-haptoglobin antibody, wherein said lectin specifically binds to  $\alpha$  2,6-sialyl residues of an  $\alpha$  2,6-sialyl residue-containing glycoprotein.
5. A method for diagnosing Alzheimer's disease, which

comprises detecting or measuring an  $\alpha$  2,6-sialyl residue-containing glycoprotein contained in a human-derived sample, using a lectin that specifically binds said  $\alpha$  2,6-sialyl residue.

6. The method according to claim 5, wherein the  $\alpha$  2,6-sialyl residue-containing glycoprotein is a 180-kD glycoprotein, a 95-kD glycoprotein, or a 40-kD glycoprotein.
7. The method according to claim 5 or 6, wherein a 40-kD glycoprotein that contains  $\alpha$  2,6-sialyl residue is detected or measured by using an anti-haptoglobin antibody.
8. The method according to claim 5, wherein a 40-kD glycoprotein that contains  $\alpha$  2,6-sialyl residue is detected or measured by sandwich assay using lectin and an anti-haptoglobin antibody, wherein said lectin specifically binds to  $\alpha$  2,6-sialyl residues of an  $\alpha$  2,6-sialyl residue-containing glycoprotein.
9. The method according to any of claims 5 to 8, wherein the human-derived sample is a blood sample.

#### Patentansprüche

1. *In vitro* Verwendung eines Agens zur Diagnose der Alzheimer'schen Krankheit, wobei das Agens Lektin umfasst, zum Nachweis eines  $\alpha$  2,6-Sialyl-Rest enthaltenden Glykoproteins, wobei das Lektin spezifisch an  $\alpha$  2,6-Sialyl-Reste eines  $\alpha$  2,6-Sialyl-Rest enthaltenden Glykoproteins bindet.
2. Die Verwendung gemäß Anspruch 1, wobei das  $\alpha$  2,6-Sialyl-Rest enthaltende Glykoprotein ein 180 kD Glykoprotein, ein 95 kD Glykoprotein oder ein 40 kD Glykoprotein ist.
3. Die Verwendung gemäß Anspruch 1, wobei das Agens weiterhin einen Anti-Haptoglobin-Antikörper zum Nachweis eines 40 kD Glykoproteins enthält, das  $\alpha$  2,6-Sialyl-Reste enthält.
4. Ein Kit zur Diagnose der Alzheimer'schen Krankheit, welcher Lektin und einen Anti-Haptoglobin-Antikörper enthält, wobei das Lektin spezifisch an  $\alpha$  2,6-Sialyl-Reste eines  $\alpha$  2,6-Sialyl-Rest enthaltenden Glykoproteins bindet.
5. Ein Verfahren zur Diagnose der Alzheimer'schen Krankheit, das den Nachweis oder das Messen eines  $\alpha$  2,6-Sialyl-Rest enthaltenden Glykoproteins umfasst, das in einer aus Menschen stammenden Probe enthalten ist, unter Verwendung eines Lektins, das spezifisch die  $\alpha$  2,6-Sialyl-Reste bindet.

6. Das Verfahren gemäß Anspruch 5, wobei das  $\alpha$  2,6-Sialyl-Rest enthaltende Glykoprotein ein 180 kD Glykoprotein, ein 95 kD Glykoprotein oder ein 40 kD Glykoprotein ist.

7. Das Verfahren gemäß Anspruch 5 oder 6, wobei ein 40 kD Glykoprotein, das  $\alpha$  2,6-Sialyl-Reste enthält, unter Verwendung eines Anti-Haptoglobin-Antikörpers nachgewiesen oder gemessen wird.

8. Das Verfahren gemäß Anspruch 5, wobei ein 40 kD Glykoprotein, das  $\alpha$  2,6-Sialyl-Rest enthält, durch einen Sandwich-Assay nachgewiesen oder gemessen wird, unter Verwendung von Lektin und eines Anti-Haptoglobin-Antikörpers, wobei das Lektin spezifisch an  $\alpha$  2,6-Sialyl-Reste eines  $\alpha$  2,6-Sialyl-Rest enthaltenden Glykoproteins bindet.

9. Das Verfahren gemäß irgend einem der Ansprüche 5 bis 8, wobei die aus Menschen stammende Probe eine Blutprobe ist.

#### Revendications

1. Utilisation *in vitro* d'un agent destiné au diagnostic de la maladie d'Alzheimer, ledit agent comprenant de la lectine utilisée pour détecter une glycoprotéine contenant des résidus  $\alpha$ -2,6-sialyle, où ladite lectine se lie spécifiquement aux résidus  $\alpha$ -2,6-sialyle d'une glycoprotéine contenant des résidus  $\alpha$ -2,6-sialyle.
2. Utilisation selon la revendication 1, où la glycoprotéine contenant des résidus  $\alpha$ -2,6-sialyle est une glycoprotéine de 180 kD, une glycoprotéine de 95 kD ou une glycoprotéine de 40 kD.
3. Utilisation selon la revendication 1, où ledit agent comprend en outre un anticorps anti-haptoglobine utilisé pour détecter une glycoprotéine de 40 kD qui contient des résidus  $\alpha$ -2,6-sialyle.
4. Kit de diagnostic de la maladie d'Alzheimer, qui comprend de la lectine et un anticorps anti-haptoglobine, où ladite lectine se lie spécifiquement aux résidus  $\alpha$ -2,6-sialyle d'une glycoprotéine contenant des résidus  $\alpha$ -2,6-sialyle.
5. Procédé de diagnostic de la maladie d'Alzheimer, qui comprend la détection ou la mesure d'une glycoprotéine contenant des résidus  $\alpha$ -2,6-sialyle contenue dans un échantillon d'origine humaine, en utilisant une lectine qui se lie spécifiquement auxdits résidus  $\alpha$ -2,6-sialyle.
6. Procédé selon la revendication 5, dans lequel une glycoprotéine contenant des résidus  $\alpha$ -2,6-sialyle est une glycoprotéine de 180 kD, une glycoprotéine

de 95 kD ou d'une glycoprotéine de 40 kD.

7. Procédé selon la revendication 5 ou 6, dans lequel une glycoprotéine de 40 kD qui contient des résidus  $\alpha$ -2,6-sialyle est détectée ou mesurée en utilisant un anticorps anti-haptoglobine. 5
8. Procédé selon la revendication 5, dans lequel une glycoprotéine de 40 kD qui contient des résidus  $\alpha$ -2,6-sialyle est détectée ou mesurée par un test « sandwich » en utilisant de la lectine et un anticorps anti-haptoglobine, où ladite lectine se lie spécifiquement aux résidus  $\alpha$ -2,6-sialyle d'une glycoprotéine contenant des résidus  $\alpha$ -2,6-sialyle. 10  
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9. Procédé selon l'une quelconque des revendications 5 à 8, dans lequel l'échantillon d'origine humaine est un échantillon de sang. 20

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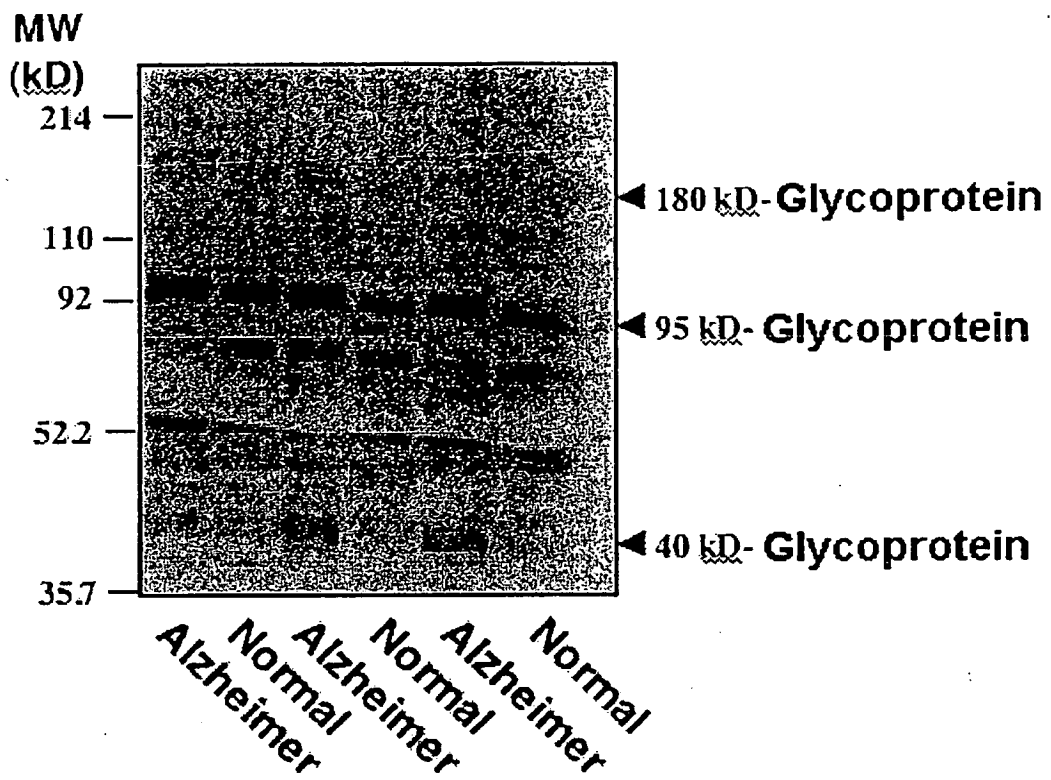
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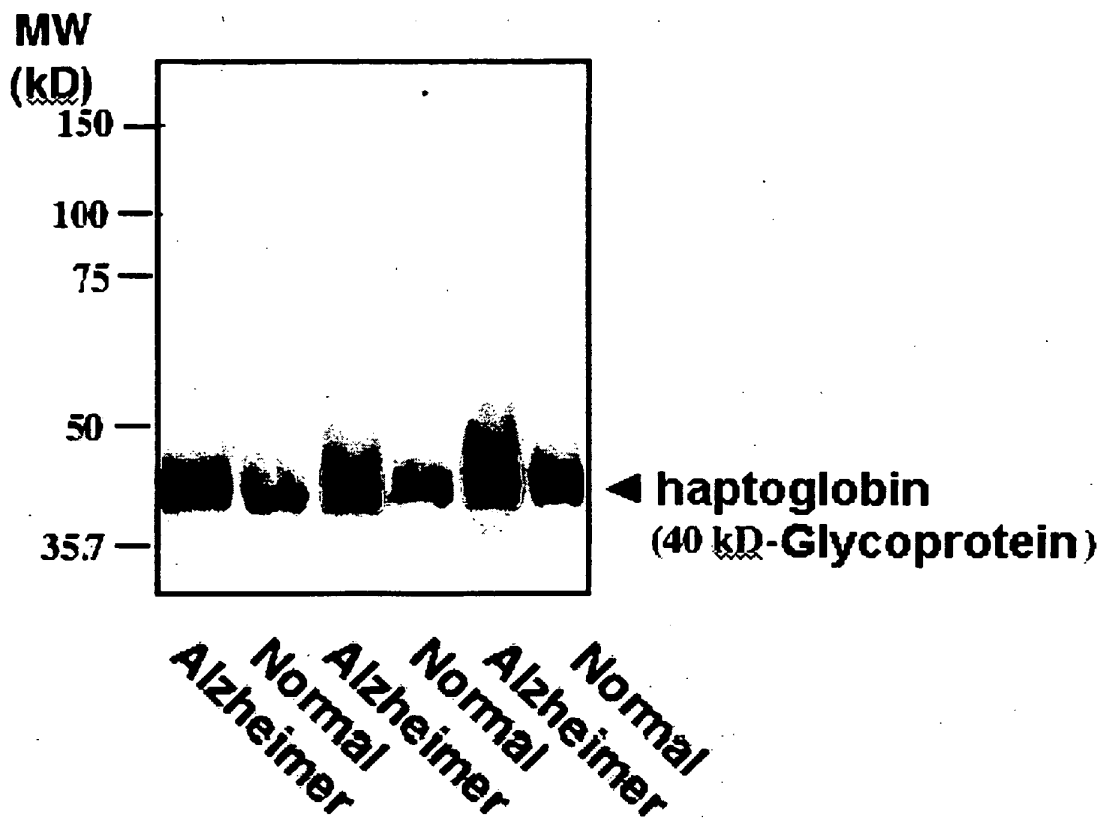
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[Fig. 1]



[Fig.2]



**REFERENCES CITED IN THE DESCRIPTION**

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**Patent documents cited in the description**

- JP 2003382374 A [0004]
- WO 2005047897 A [0004]

**Non-patent literature cited in the description**

- **Selkoe, D. J.** *Physiol. Rev.*, 2001, vol. 81, 741-766 [0002]
- **Iwata, N. et al.** *Science*, 2001, vol. 292, 1550-1552 [0002]
- **De Strooper, B. et al.** *Nature (London)*, 1998, vol. 391, 387-390 [0002]
- **Wolfe, M. S. et al.** *Nature (London)*, 1990, vol. 398, 513-517 [0002]
- **Buxbaum, J. D. et al.** *J. Biol. Chem.*, 1998, vol. 273, 27765-27767 [0002]
- **Lammich, S. et al.** *Proc. Natl. Acad. Sci. USA*, 1999, vol. 96, 3922-3927 [0002]
- **Johnson et al.** *Applied and Theoretical Electrophoresis*, 1992, vol. 3 (2), 47-53 [0005]
- **Yasukawa et al.** *Glycobiology*, 2005, vol. 15 (9), 827-837 [0006]
- **G. Kohler et al.** *Nature*, 1975, vol. 256, 495 [0026]

专利名称(译)	用血清糖蛋白作为生物标志物诊断阿尔茨海默病的方法		
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#### 摘要(译)

本发明的一个目的是检测血液中含有 $\alpha$ 2,6-唾液酸残基的糖蛋白代谢的变化，从而提供一种诊断散发性阿尔茨海默病的药剂和方法。本发明提供用于诊断阿尔茨海默病的药剂，其包含用于检测含有 $\alpha$ 2,6-唾液酸残基的糖蛋白的凝集素。

