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(54) **METHOD OF STABILIZING PROTEIN**

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(57) **ABSTRACT**

The present inventors revealed that deamidation of an antibody can be suppressed without influencing its activity by substituting a glycine that is located adjacent to an asparagine with another amino acid.

FIG. 1

HEAVY CHAIN

QVQLLESGAVLARPGTSVKISCKASGFNIK DYYMH WVKQRPGQGLEWIG GNDPANGHSMYDPKFQG

FR1 CDR1 FR2 CDR2

RVTITADTSTSTVFMELSSLRSEDTAVYYCAR DSGYAMDY WGQGTLVTVSS

FR3 CDR3 FR4

ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTK

VDKRVESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVS

VLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP

PVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK

LIGHT CHAIN

DIQMTQSPSSLSASVGDRVTITCKASQDIKSFLSWYQQKPEKAPKSLIYYATSLADGVPSRFSGSGSGTDYTLTISSLQPEDFATYYCLQHGES

PYTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADY

EKHKVYACEVTHQGLSSPVTKSFNRGEC

FIG. 2

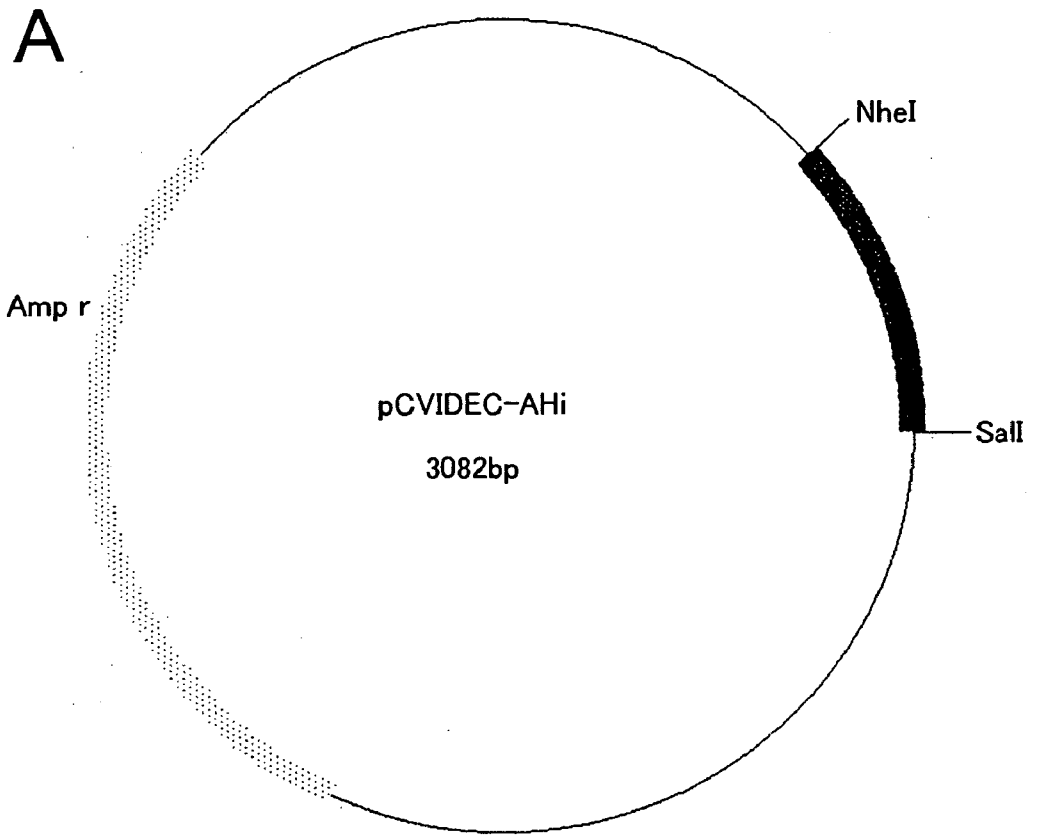
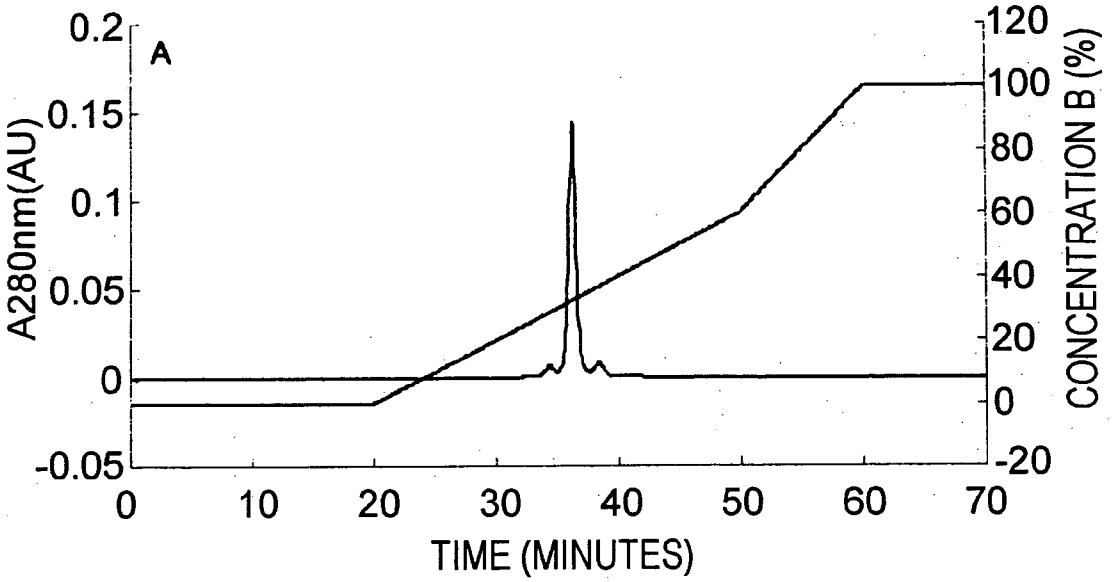

A
NheI
Amp r
pCVIDEC-AHi
3082bp
SalI
B
HEAVY CHAIN VARIABLE REGION OF
ANTI-HUMAN TF ANTIBODY (425 bp)
EcoT22I
SalI
MroI
XbaI
BalI
NheI
SGFNIKDYYMH
LEWIIGGNDPANG
N28D
N51D
N54D

FIG. 3


A
A280nm(AU)
0.2
0.15
0.1
0.05
0
-0.05
0
10
20
30
40
50
60
70
TIME (MINUTES)
120
100
80
60
40
20
0
-20
CONCENTRATION B (%)

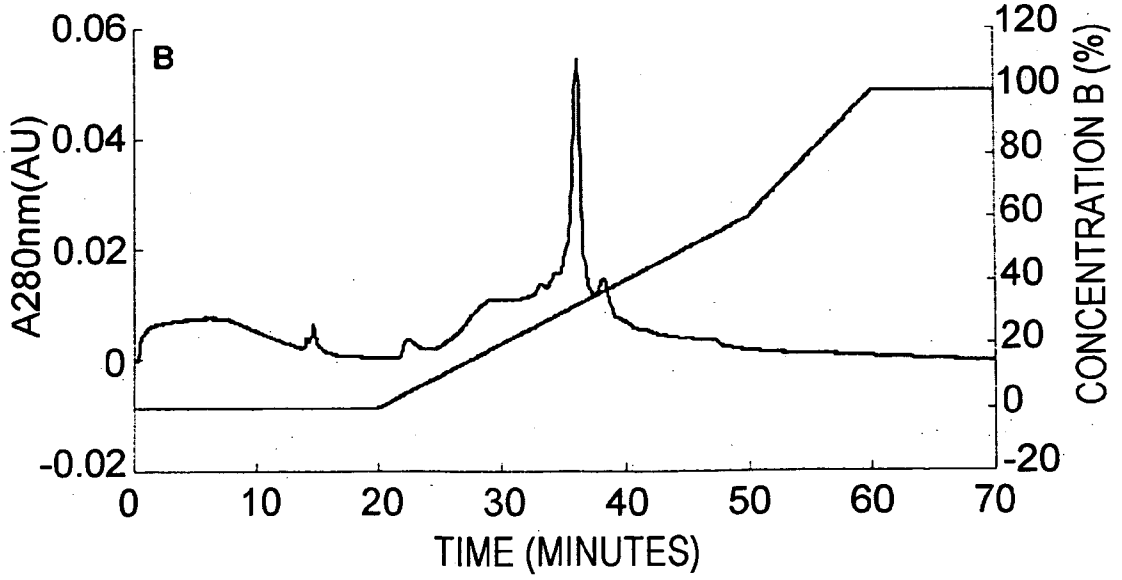
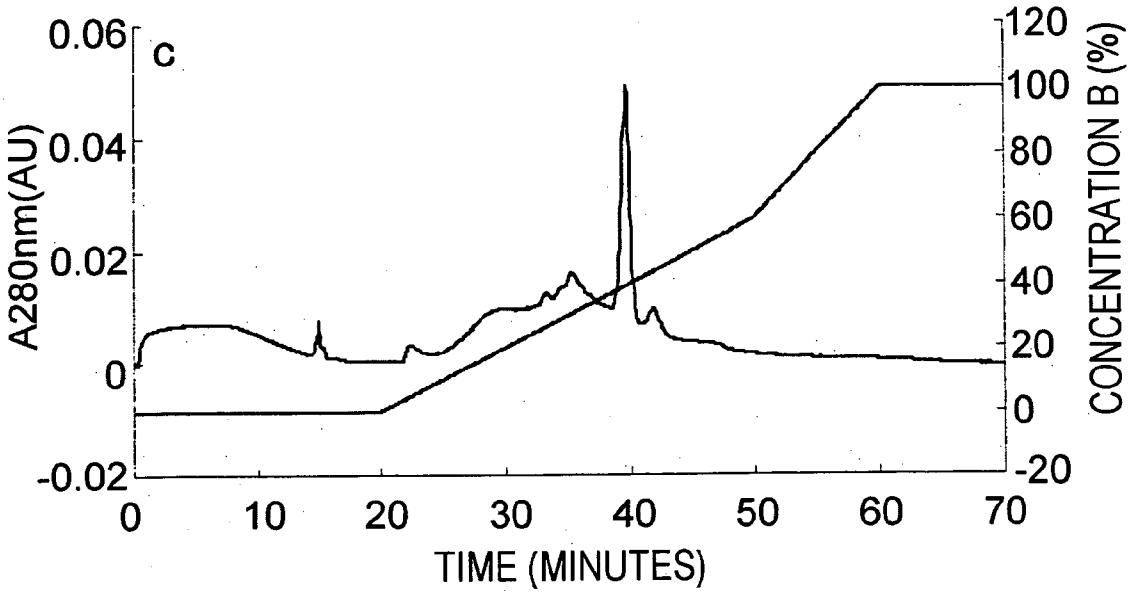

B
A280nm(AU)
0.06
0.04
0.02
0
-0.02
0
10
20
30
40
50
60
70
TIME (MINUTES)
120
100
80
60
40
20
0
-20
CONCENTRATION B (%)

FIG. 4


C
A280nm(AU)
0.06
0.04
0.02
0
-0.02
CONCENTRATION B (%)
120
100
80
60
40
20
0
-20
0
10
20
30
40
50
60
70
TIME (MINUTES)

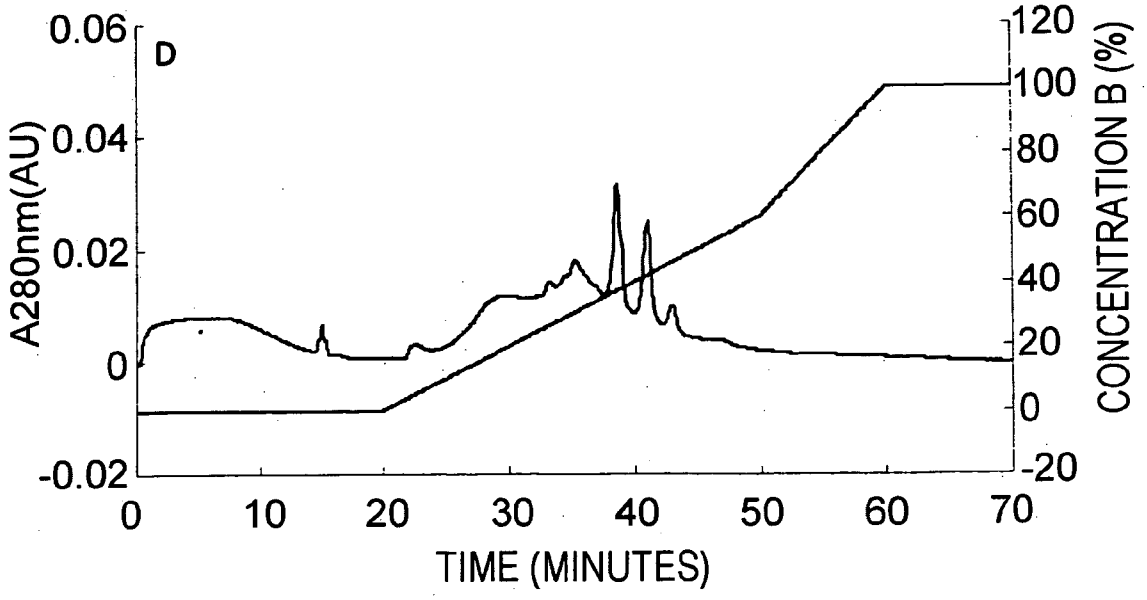
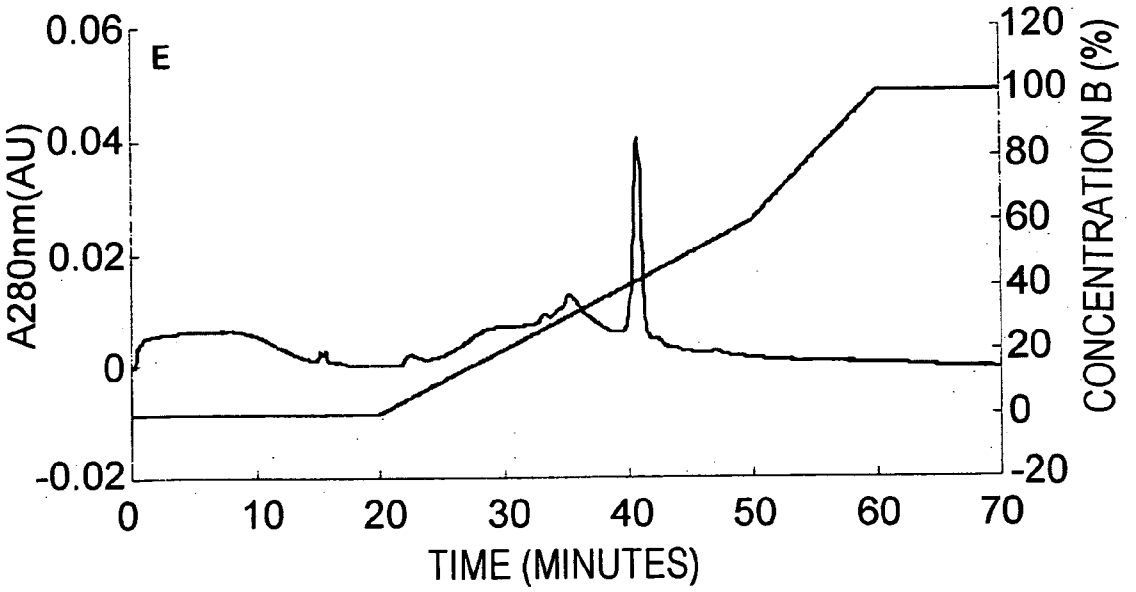

D
A280nm(AU)
0.06
0.04
0.02
0
-0.02
CONCENTRATION B (%)
120
100
80
60
40
20
0
-20
0
10
20
30
40
50
60
70
TIME (MINUTES)

FIG. 5


E
A280nm(AU)
0.06
0.04
0.02
0
-0.02
0
10
20
30
40
50
60
70
TIME (MINUTES)
120
100
80
60
40
20
0
-20
CONCENTRATION B (%)

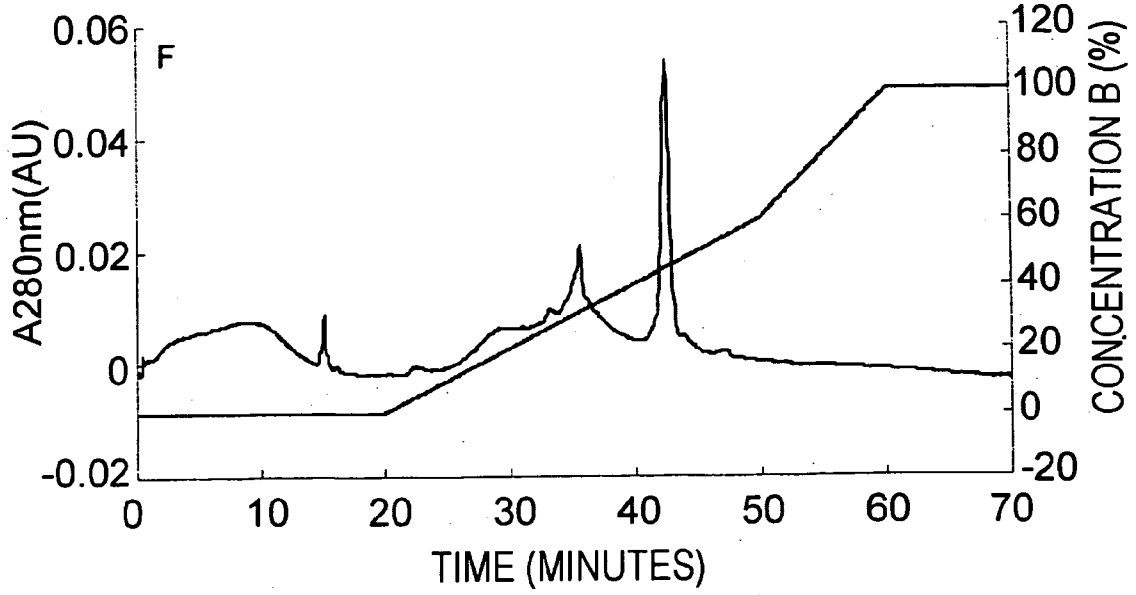

F
A280nm(AU)
0.06
0.04
0.02
0
-0.02
0
10
20
30
40
50
60
70
TIME (MINUTES)
120
100
80
60
40
20
0
-20
CONCENTRATION B (%)

FIG. 6

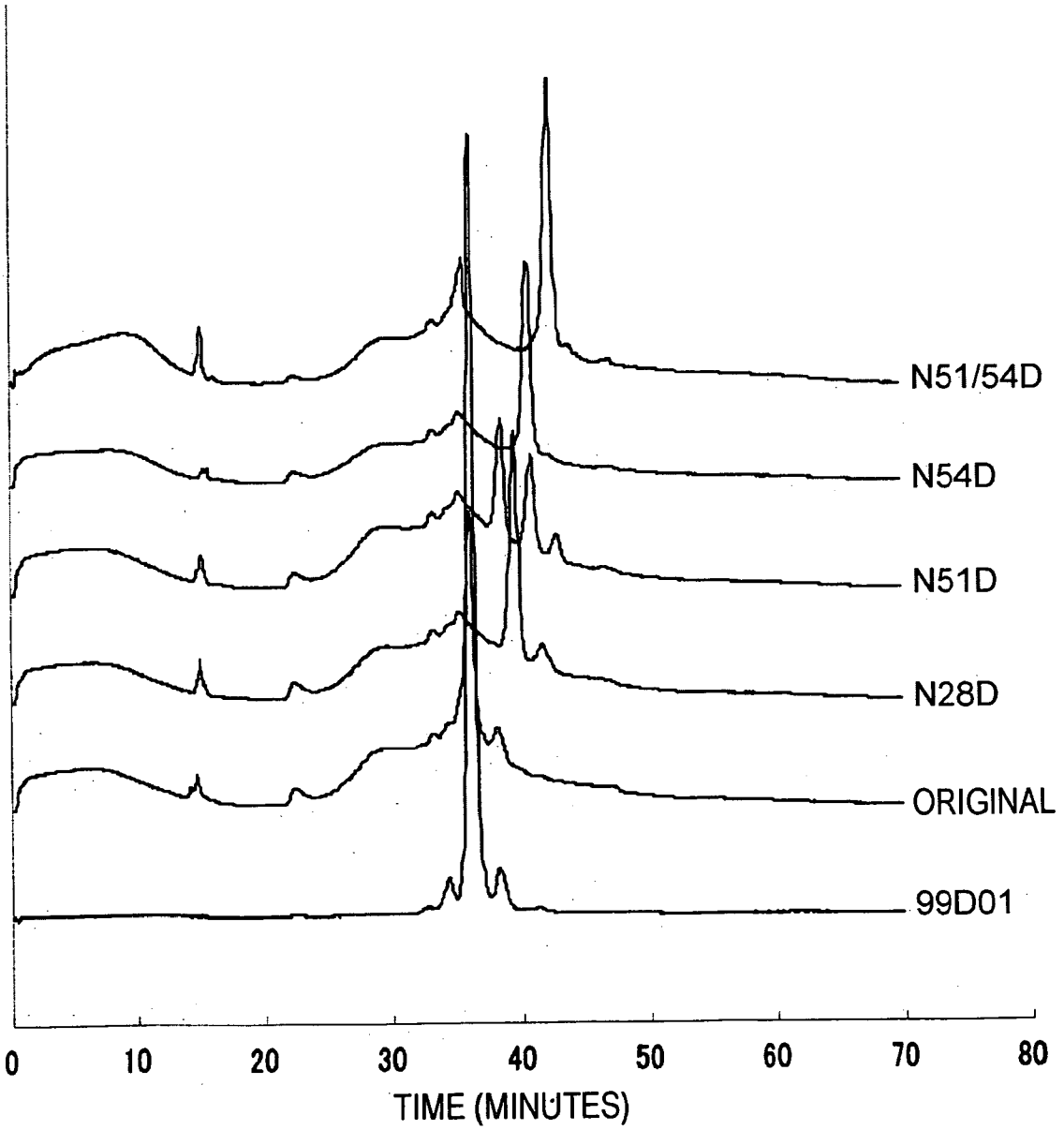

N51/54D
N54D
N51D
N28D
ORIGINAL
99D01
0
10
20
30
40
50
60
70
80
TIME (MINUTES)

FIG. 7

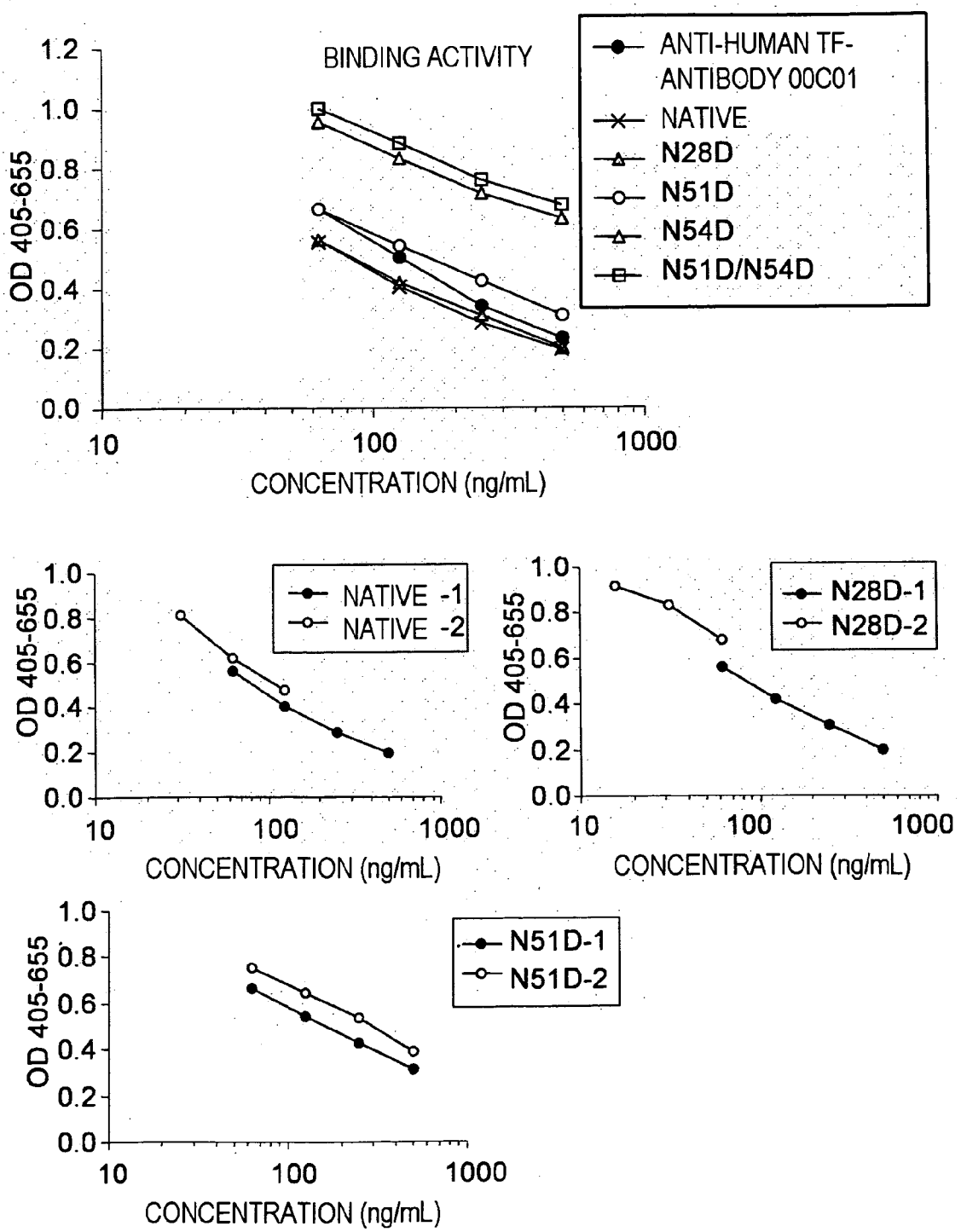

BINDING ACTIVITY
OD 405-655
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
100
1000
CONCENTRATION (ng/mL)
ANTI-HUMAN TF-ANTIBODY 00C01
NATIVE
N28D
N51D
N54D
N51D/N54D
NATIVE -1
NATIVE -2
N28D-1
N28D-2
N51D-1
N51D-2

FIG. 8

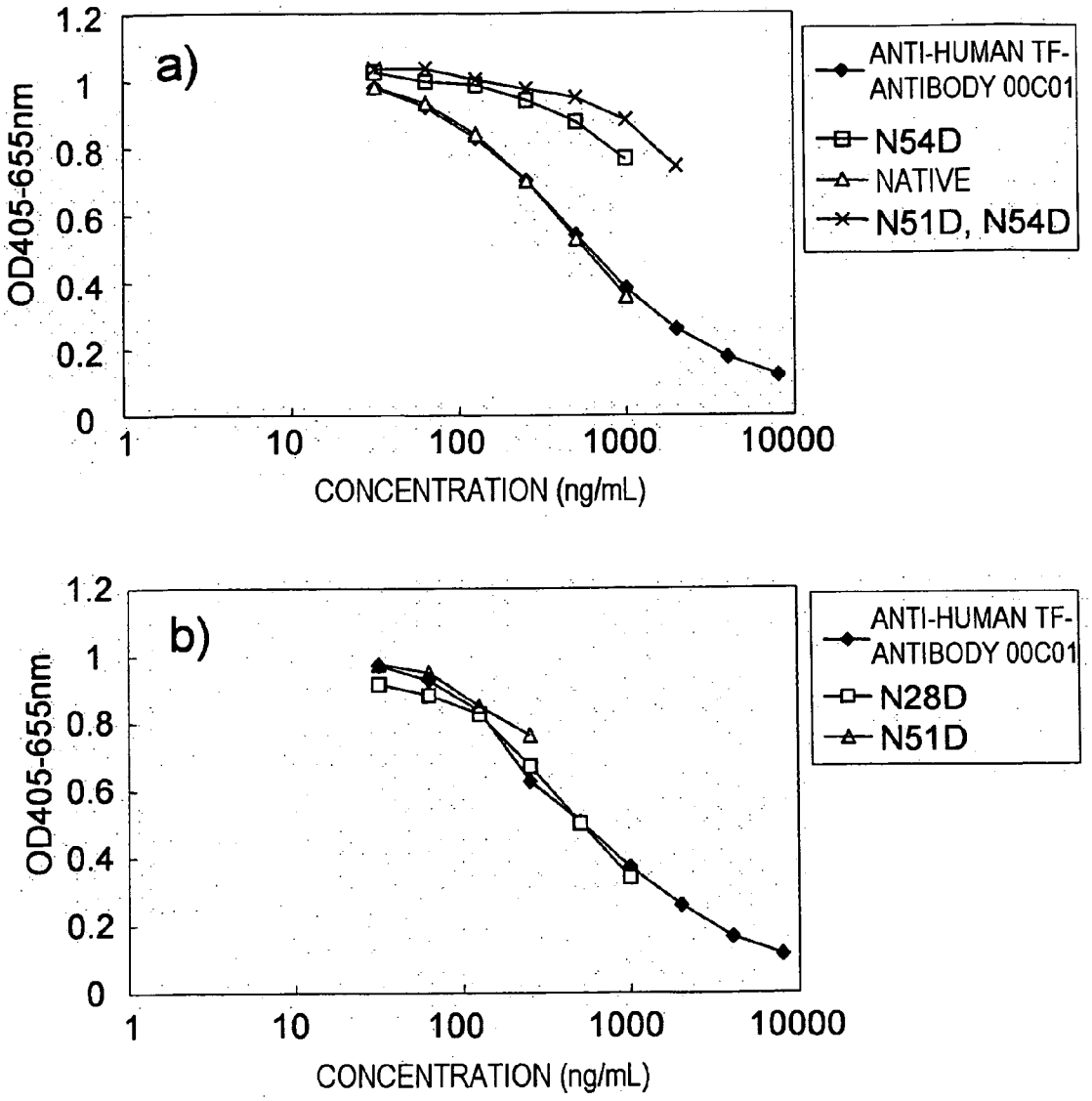

a)
OD405-655nm
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
1000
10000
CONCENTRATION (ng/mL)
ANTI-HUMAN TF-ANTIBODY 00C01
N54D
NATIVE
N51D, N54D
b)
OD405-655nm
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
1000
10000
CONCENTRATION (ng/mL)
ANTI-HUMAN TF-ANTIBODY 00C01
N28D
N51D

FIG. 9

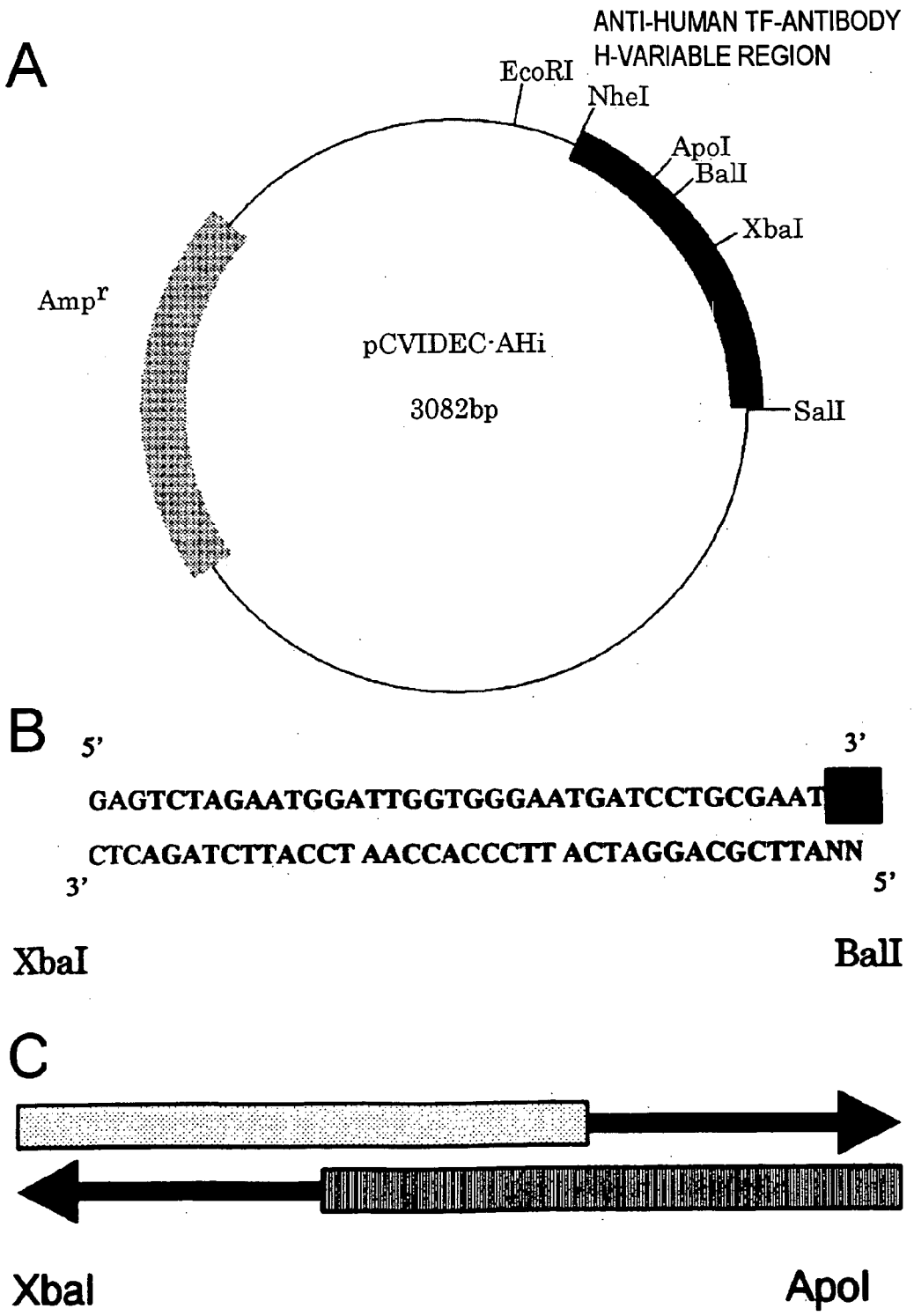
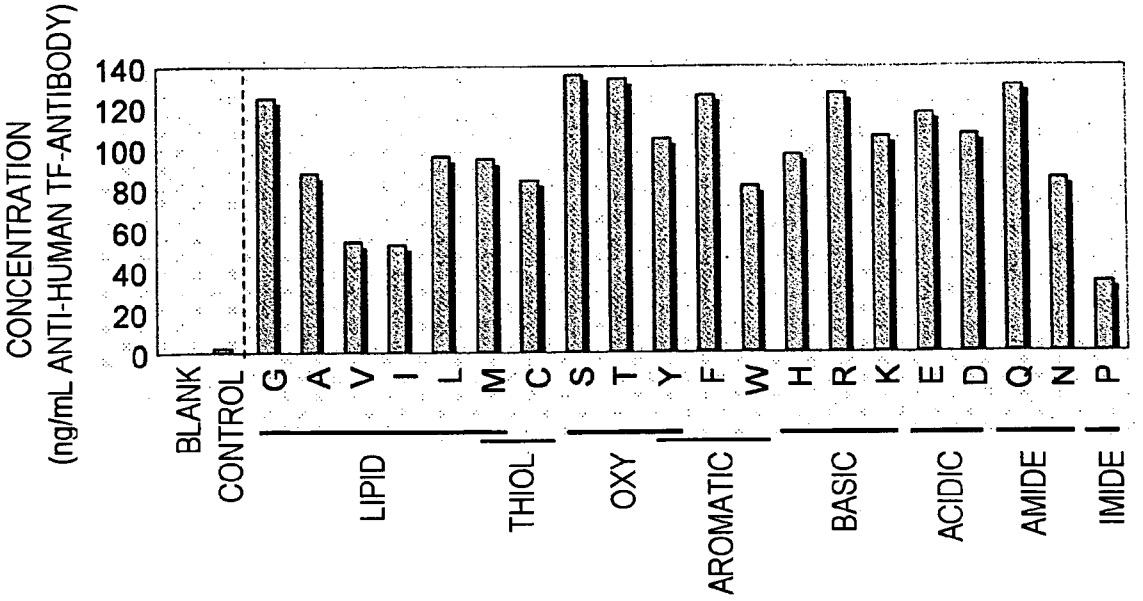

A
ANTI-HUMAN TF-ANTIBODY
H-VARIABLE REGION
EcoRI
NheI
ApoI
BalI
XbaI
SalI
Amp^r
pCVIDEC-AHi
3082bp
B
5'
3'
GAGTCTAGAATGGATTGGTGGGAATGATCCTGCGAAT
CTCAGATCTTACCT AACCACCCTT ACTAGGACGCTTANN
3'
5'
XbaI
BalI
C
Xbal
Apol

FIG. 10



SAMPLE: 100 ng/mL

FIG. 11

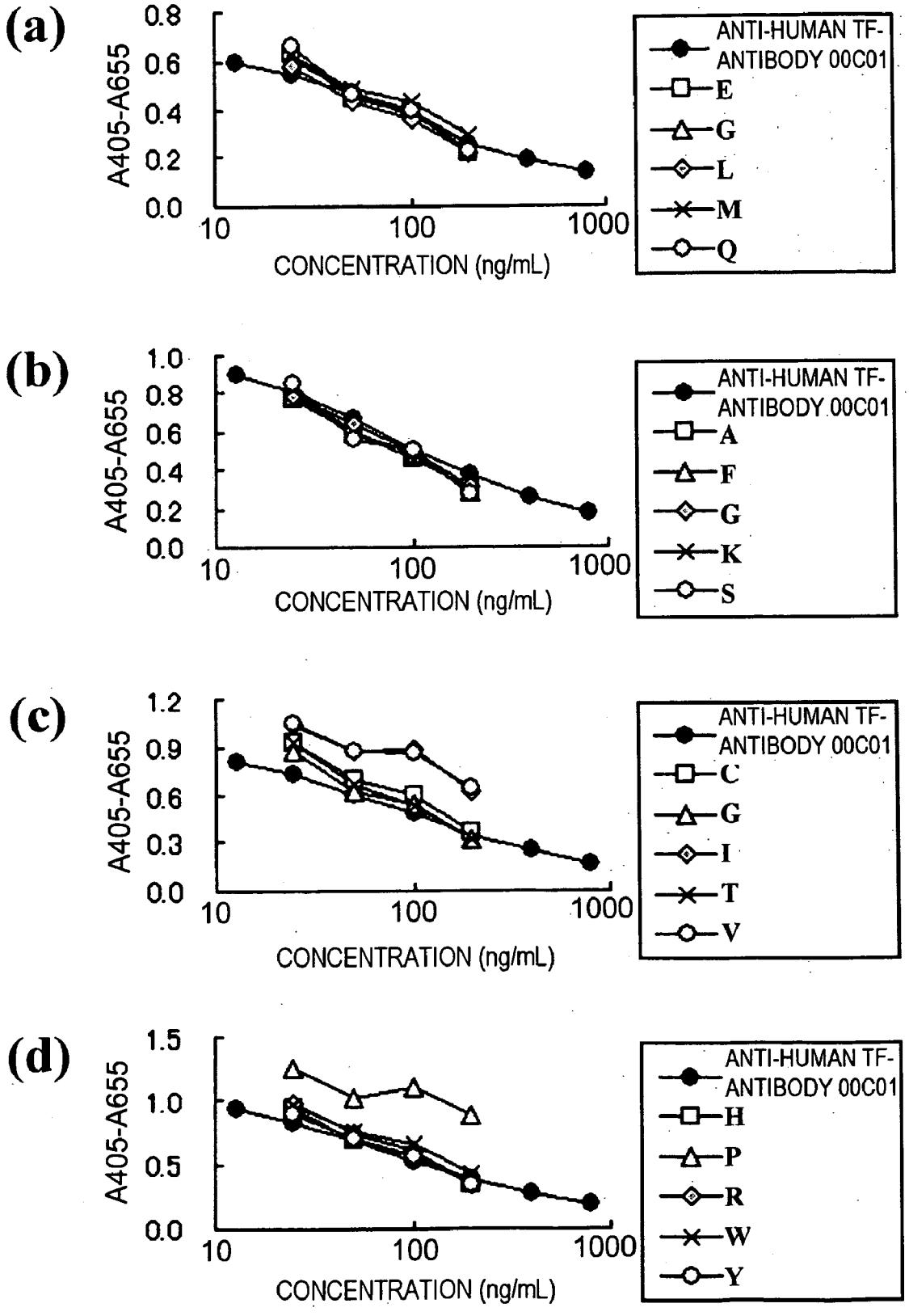

(a)
A405-A655
0.8
0.6
0.4
0.2
0.0
10
100
1000
CONCENTRATION (ng/mL)
ANTI-HUMAN TF-ANTIBODY 00C01
E
G
L
M
Q
(b)
A405-A655
1.0
0.8
0.6
0.4
0.2
0.0
10
100
1000
CONCENTRATION (ng/mL)
ANTI-HUMAN TF-ANTIBODY 00C01
A
F
G
K
S
(c)
A405-A655
1.2
0.9
0.6
0.3
0.0
10
100
1000
CONCENTRATION (ng/mL)
ANTI-HUMAN TF-ANTIBODY 00C01
C
G
I
T
V
(d)
A405-A655
1.5
1.0
0.5
0.0
10
100
1000
CONCENTRATION (ng/mL)
ANTI-HUMAN TF-ANTIBODY 00C01
H
P
R
W
Y

FIG. 12

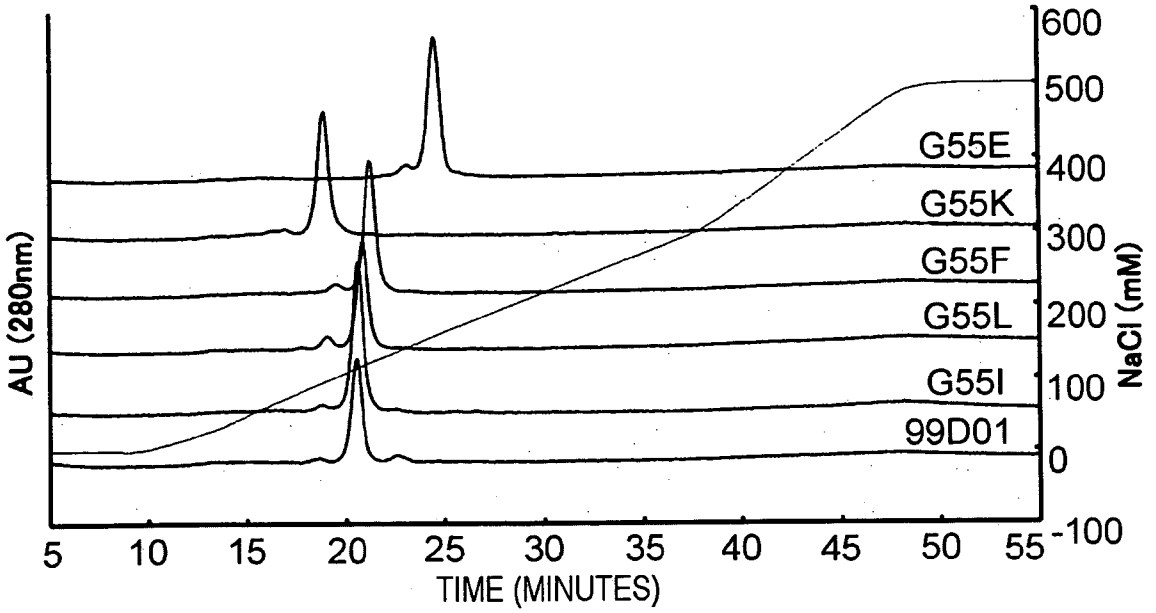

AU (280nm)
G55E
G55K
G55F
G55L
G55I
99D01
600
500
400
300
200
100
0
-100
NaCl (mM)
5
10
15
20
25
30
35
40
45
50
55
TIME (MINUTES)

FIG. 13

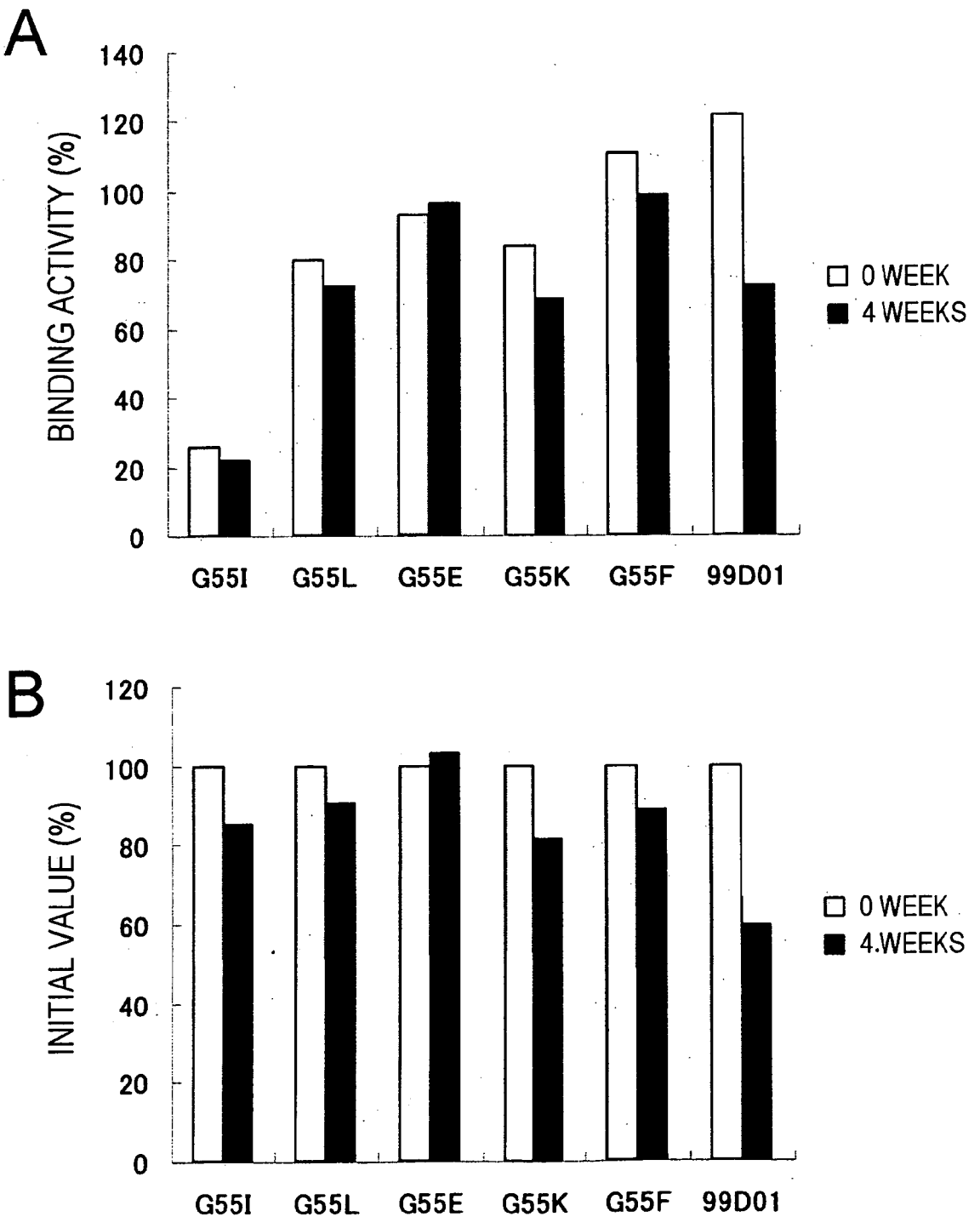

A
BINDING ACTIVITY (%)
140
120
100
80
60
40
20
0
G55I
G55L
G55E
G55K
G55F
99D01
0 WEEK
4 WEEKS
B
INITIAL VALUE (%)
120
100
80
60
40
20
0
G55I
G55L
G55E
G55K
G55F
99D01
0 WEEK
4.WEEKS

FIG. 14

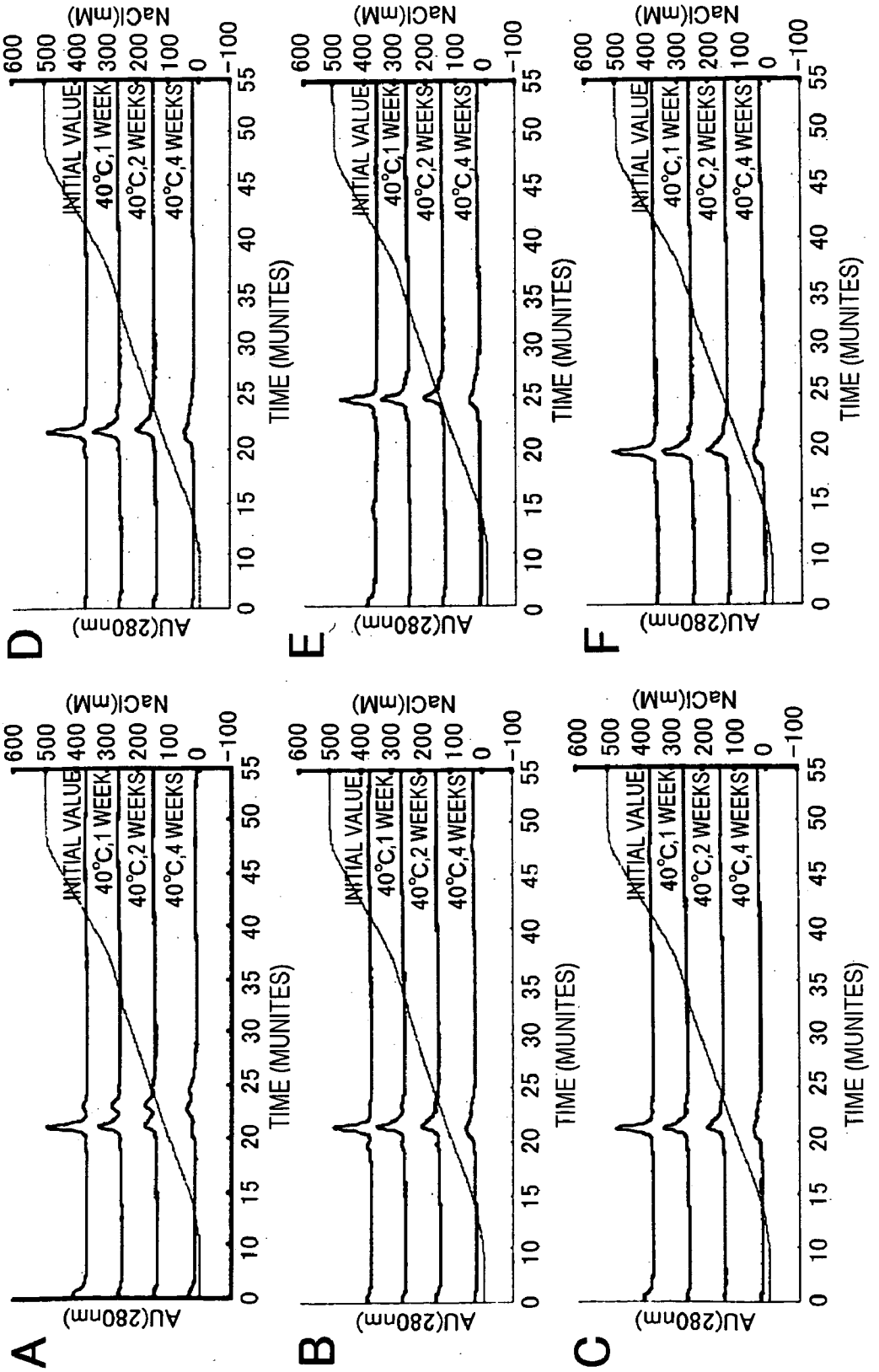

A
B
C
D
E
F
AU(280nm)
NaCl(mM)
TIME (MUNITES)
INITIAL VALUE
40°C,1 WEEK
40°C,2 WEEKS
40°C,4 WEEKS
600
500
400
300
200
100
0
-100
0 10 15 20 25 30 35 40 45 50 55

FIG. 15

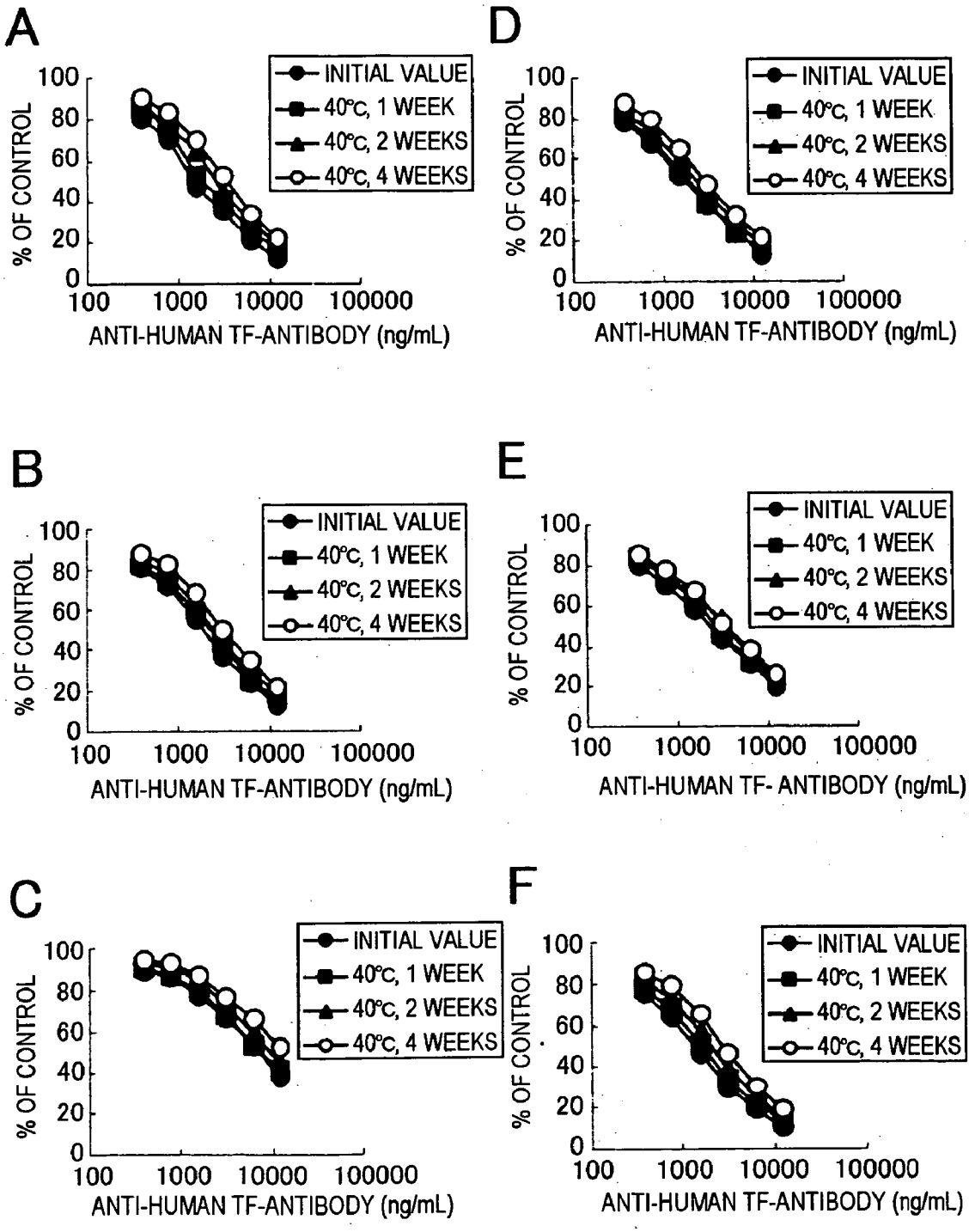

A
D
B
E
C
F
% OF CONTROL
100
80
60
40
20
0
100
1000
10000
100000
ANTI-HUMAN TF-ANTIBODY (ng/mL)
ANTI-HUMAN TF- ANTIBODY (ng/mL)
INITIAL VALUE
40°C, 1 WEEK
40°C, 2 WEEKS
40°C, 4 WEEKS

FIG. 16

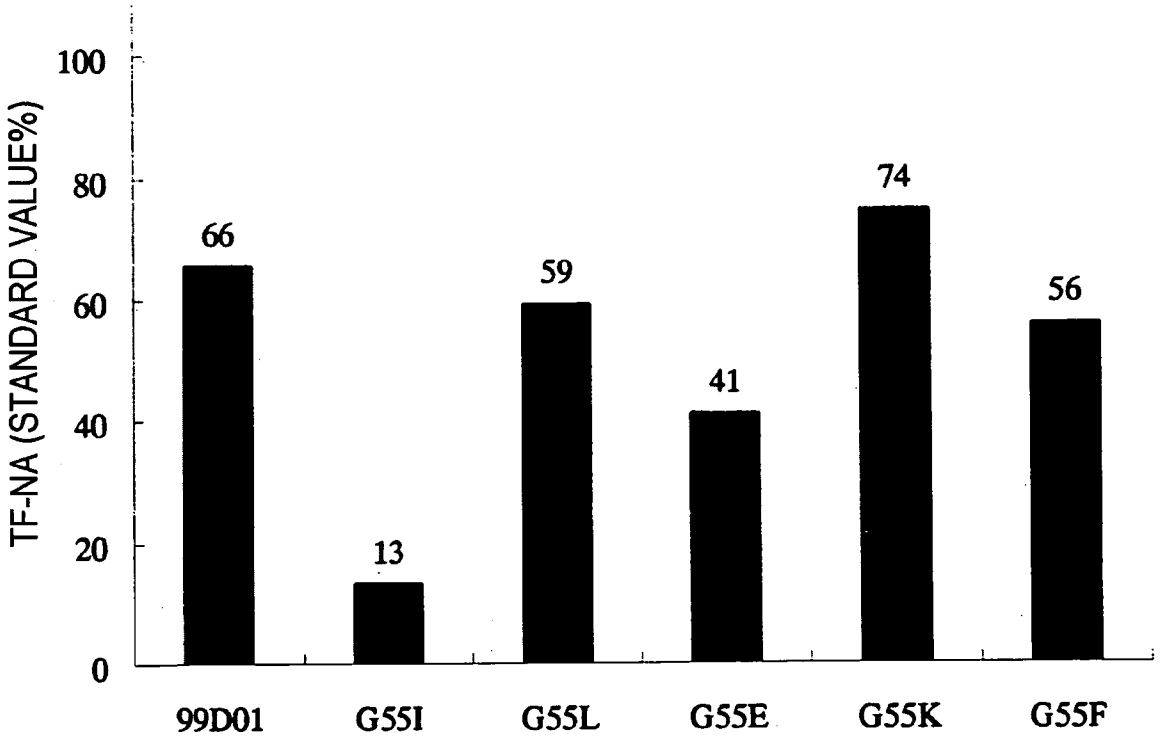
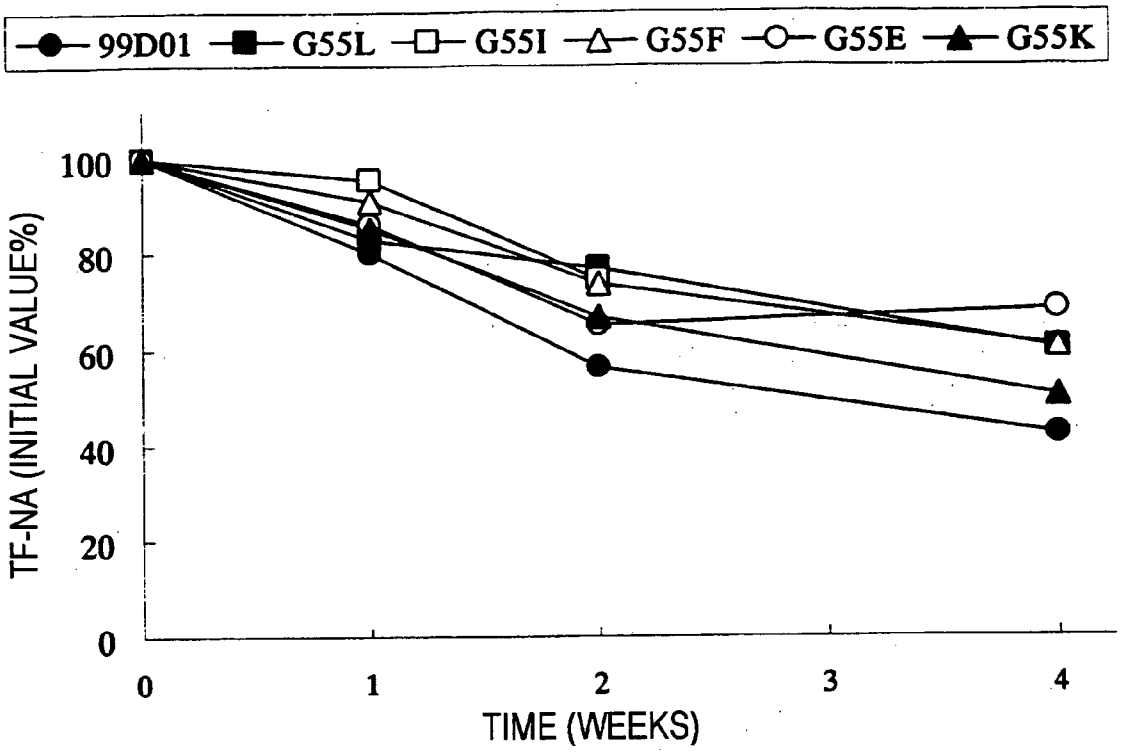

TF-NA (STANDARD VALUE%)
100
80
60
40
20
0
66
13
59
41
74
56
99D01
G55I
G55L
G55E
G55K
G55F

FIG. 17


99D01
G55L
G55I
G55F
G55E
G55K
TF-NA (INITIAL VALUE%)
100
80
60
40
20
0
0
1
2
3
4
TIME (WEEKS)

METHOD OF STABILIZING PROTEIN

TECHNICAL FIELD

[0001] The present invention relates to a method for improving protein stability. Specifically, the present invention relates to a method for stabilizing proteins comprising the step of substituting the amino acid that is located adjacent to the amino acid being deamidated in a protein with another amino acid.

BACKGROUND ART

[0002] Gradual deamidation of amino acids, such as asparagine, in proteins over time is mentioned as a cause of the reduction in protein stability. When proteins, particularly antibodies, are used as pharmaceutical agents for various diseases, they are required to be stable over a long period. However, the activity of antibody decreases with time. The cause for reduction in activity varies in antibodies, and deamidation of amino acids, such as asparagine, comprised in the antibody is also mentioned as one of the causes.

[0003] Therefore, proteins can be stabilized by suppressing deamidation of asparagines. Thus, research on suppressing deamidation of asparagine has been conducted. The substitution of asparagine with another amino acid by site-directed mutagenesis is considered the most certain method to prevent deamidation of proteins. However, this substitution has the potential to influence protein activity. For example, when the asparagine is located in the complementary determining region (CDR) of an antibody, such substitution is reported to affect the antibody binding affinity (Presta L. et al., Thromb. Haemost. 85: 379-389, 2001). An anti-human tissue factor (TF) antibody that is expected to inhibit thrombus formation without inhibiting the extrinsic blood coagulation reaction via the inhibition of Factor X activation mediated by TF in the intrinsic blood coagulation reaction is known in the art (WO 99/51743). However, this antibody has not been formulated as a pharmaceutical preparation and its activity reduces over time under antibody destabilizing conditions. The deamidation of anti-human TF antibody is supposed to be a factor of such reduction.

[0004] Thus, a method to suppress deamidation of asparagine without influencing antibody activity has been desired in the art.

DISCLOSURE OF THE INVENTION

[0005] Reduction in protein activity is a very important problem from the medical and pharmaceutical perspectives. Particularly, antibodies that are stable for a long time and which can be used as pharmaceutical agents are clinically desired. To stabilize antibodies, it is particularly required to suppress deamidation over time of amino acids such as asparagine, mainly, those readily deamidated in Asn-Gly sequences.

[0006] Conventionally, methods to suppress deamidation by altering amino acids in proteins is a useful technique to improve the value, quality and such of pharmaceuticals. Such methods increase the option in the formulation of pharmaceutical preparations, and thus facilitate application of the proteins to various drug forms and administration routes. Therefore, the purpose of the present invention is to provide a method to suppress deamidation of asparagine without influencing the activity of proteins, particularly antibodies.

[0007] The present inventors diligently conducted research focusing on anti-human TF antibody, which use as a pharmaceutical is expected in the art. The antibody was used as an example of a protein for developing a method to suppress deamidation of asparagine without affecting the protein activity. First, a mutated anti-human TF antibody was expressed as a recombinant wherein asparagine that may be deamidated yet existing in the CDR, is substituted with aspartic acid. The TF binding activity of anti-human TF antibody was suggested to decrease significantly due to the deamidation of Asn54 existing in the CDR2 region of the anti-human TF antibody heavy chain (H chain). The amino acid adjacent to Asn54 in the CDR2 region of anti-human TF antibody heavy chain is Gly55. These two amino acids form a primary sequence Asn-Gly that is easily deamidated. Therefore, the possibility to suppress deamidation of Asn54 by substituting this Gly55 with another amino acid was considered. Thus, the present inventors prepared mutants wherein the glycine adjacent to the asparagine was substituted with other amino acids to measure their binding activities. As a result, it was discovered that the substitution of glycine that is located adjacent to asparagine with other amino acids did not reduce the activity, and also suppressed the known instability due to deamidation.

[0008] Thus, the present inventors found that antibody activity is uninfluenced by the substitution of glycine that is located adjacent to asparagine with other amino acids, instead of the substitution of the asparagine itself, and thereby completed the present invention.

[0009] Specifically, the present invention provides the following:

[0010] (1) a method for stabilizing a protein, which comprises the step of substituting an amino acid that is located adjacent to an amino acid being deamidated with another amino acid;

[0011] (2) the method for stabilizing a protein of (1), wherein the amino acid being deamidated is asparagine;

[0012] (3) the method for stabilizing a protein of (1), wherein the amino acid that is located adjacent to the C-terminal side of the amino acid being deamidated is glycine;

[0013] (4) the method for stabilizing a protein of any one of (1) to (3), wherein the protein is an antibody;

[0014] (5) the method for stabilizing a protein of (4), wherein the antibody is humanized antibody;

[0015] (6) the method for stabilizing a protein of (4) or (5), wherein the amino acid being deamidated exists in the complementary determining region (CDR);

[0016] (7) the method for stabilizing a protein of (6), wherein the complementary determining region (CDR) is CDR2;

[0017] (8) the method for stabilizing a protein of any one of (1) to (3), wherein the protein is an antigen binding protein;

[0018] (9) the method for stabilizing a protein of any one of (1) to (3), wherein the protein belongs to the immunoglobulin superfamily;

[0019] (10) the method for stabilizing a protein of any one of (1) to (3), wherein the protein is a pharmaceutical agent;

[0020] (11) a protein stabilized by the method of any one of (1) to (10); and

[0021] (12) the stabilized protein of (11) whose antigen binding activity is 70% or more of the activity before the amino acid substitution.

[0022] The terms described in the specification are defined as follows. However, it should be understood that the definitions are provided to facilitate understanding of the terms used herein and are not to be construed as limiting the present invention.

[0023] The term "protein" herein refers to recombinant proteins, natural proteins and synthetic peptides prepared by artificially combining amino acids, which proteins and peptides consist of five amino acids or more. Proteins consist of amino acid sequences having preferably 14 residues or more, more preferably 30 residues or more, and much more preferably 50 residues or more.

[0024] The term "antibody" used in the stabilization method of the present invention is used in the broadest sense, and includes monoclonal antibodies (including full-length monoclonal antibodies), polyclonal antibodies, mutant antibodies, antibody fragments (for example, Fab, $F(ab')_2$ and Fv) and multispecific antibodies (for example, bispecific antibodies) as long as they have the desired biological activity. Antibodies (Ab) and immunoglobulins (Ig) are glycoproteins that share the same structural features. Antibodies show a specific binding ability to a certain antigen, while immunoglobulins include antibodies and other antibody-like molecules that lack antigen specificity. Natural antibodies and immunoglobulins are generally heterotetramers of about 150,000 Daltons consisting of 2 identical light chains (L chains) and 2 identical heavy chains (H chains). Each of the light chain is connected to a heavy chain through a single covalent disulfide bond. However, the number of disulfide bonds between the heavy chains varies depending on the isotype of the immunoglobulin. Both of the heavy and light chains further have intramolecular disulfide bridges at constant distance. Each of the heavy chain has a variable region (V_H) at one end and many constant regions connected thereto. Each of the light chain has a variable region (V_L) at one end and a constant region at the other end. The constant region and the variable region of the light chain are placed side-by-side to the first constant region and the variable region of the heavy chain, respectively. Specific amino acid residues are considered to form the interface of the variable region of the light and heavy chains (Chothia C. et al. J. Mol. Biol. 186: 651-663, 1985; Novotny J., Haber E., Proc. Natl. Acad. Sci. USA 82: 4592-4596, 1985).

[0025] The light chains of antibodies (immunoglobulins) derived from vertebrate species can be divided into two clearly distinct types called kappa (κ) and lambda (λ), based on the amino acid sequence of the constant region. In addition, an "immunoglobulin" can be classified into different classes based on the amino acid sequence of the constant region of the heavy chain. At least five major classes exist for immunoglobulins: IgA, IgD, IgE, IgG and IgM. Furthermore, some of them can be further classified into subclasses (isotypes), for example, IgG-1, IgG-2, IgG-3 and IgG-4, and IgA-1 and IgA-2. The heavy chain constant regions of the different classes are called α, δ, ε, γ and μ, respectively. The subunit structures and three-dimensional structures of immunoglobulins of each class are well known.

[0026] Herein, the phrase "monoclonal antibody" refers to an antibody obtained from a group of substantially homogeneous antibodies, i.e., an antibody group wherein the antibodies constituting the group are homogeneous except for naturally occurring mutants that exist in a small amount. A monoclonal antibody is highly specific and interacts with a single antigenic site. Furthermore, each monoclonal antibody targets a single antigenic determinant (epitope) on an antigen, as compared to common (polyclonal) antibody preparations that typically contain various antibodies against diverse antigenic determinants. In addition to their specificity, monoclonal antibodies are advantageous in that they are produced from hybridoma cultures not contaminated with other immunoglobulins.

[0027] The qualifier "monoclonal" indicates the characteristics of antibodies obtained from a substantially homogeneous group of antibodies, and does not require that the antibodies be produced by a particular method. For example, the monoclonal antibody used in the present invention can be produced by, for example, the hybridoma method (Kohler G. and Milstein C., Nature 256: 495-497, 1975) or the recombination method (U.S. Pat. No. 4,816,567). The monoclonal antibodies used in the present invention can be also isolated from a phage antibody library (Clackson T. et al., Nature 352: 624-628, 1991; Marks J. D. et al., J. Mol. Biol. 222:581-597, 1991). The monoclonal antibodies in the present specification particularly include "chimeric" antibodies (immunoglobulins) wherein a part of the heavy chain and/or light chain is derived from a specific species, or a specific antibody class or subclass and the remaining portion of the chain from another species, or another antibody class or subclass. Furthermore, as long as they have the desired biological activity, antibody fragments thereof are also included in the present invention (U.S. Pat. No. 4,816,567; Morrison S. L. et al., Proc. Natl. Acad. Sci. USA 81: 6851-6855, 1984).

[0028] The phrase "mutant antibody" refers to amino acid sequence variants of antibodies wherein one or more amino acid residues are altered. The "mutant antibody" herein includes variously altered amino acid variants as long as they have the same binding specificity as the original antibody. Such mutants have less than 100% homology or similarity to the amino acid sequence that has at least 75%, more preferably at least 80%, even more preferably at least 85%, still more preferably at least 90%, and most preferably at least 95% amino acid sequence homology or similarity to the amino acid sequence of the variable region of the heavy chain or light chain of an antibody. The method of the present invention is equally applicable to both polypeptides, antibodies and antibody fragments; therefore, these terms are often used interchangeably.

[0029] The phrase "antibody fragment" refers to a part of a full-length antibody and generally indicates an antigen-binding region or a variable region. For example, antibody fragments include Fab, Fab', $F(ab')_2$ and Fv fragments. Papain digestion of an antibody produces two identical antigen-binding fragments called Fab fragments each having

an antigen-binding region, and a remaining fragment called "Fc" since it crystallizes easily. On the other hand, by the digestion with pepsin, a $F(ab')_2$ fragment (which has two antigen-binding sites and can cross bind antigens) and the remaining other fragment (called pFc') are obtained. Other fragments include diabody (diabodies), linear antibodies, single-chain antibodies, and multispecific antibodies formed from antibody fragments. In this specification, "functional fragment" of an antibody indicates Fv, F(ab) and $F(ab')_2$ fragments.

[0030] Herein, an "Fv" fragment is the smallest antibody fragment and contains a complete antigen recognition site and a binding site. This region is a dimmer (V_H-V_L dimmer) wherein the variable regions of each of the heavy chain and light chain are strongly connected by a noncovalent bond. The three CDRs of each of the variable regions interact with each other to form an antigen-binding site on the surface of the V_H-V_L dimmer. Six CDRs confer the antigen-binding site of an antibody. However, a variable region (or a half of Fv which contains only three CDRs specific to an antigen) alone has also the ability to recognize and bind an antigen although its affinity is lower than the affinity of the entire binding site.

[0031] Moreover, a Fab fragment (also referred to as F(ab)) further includes the constant region of the light chain and a constant region (C_H1) of the heavy chain. An Fab' fragment differs from the Fab fragment in that it additionally has several residues derived from the carboxyl end of the heavy chain C_H1 region which contains one or more cysteines from the hinge domain of the antibody. Fab'-SH indicates an Fab' wherein one or more cysteine residues of the constant region has a free thiol-group. The F(ab') fragment is produced by the cleavage of disulfide bonds between the cystines in the hinge region of the $F(ab')_2$ pepsin digest. Other chemically bound antibody fragments are also known by those skilled in art.

[0032] The term "diabody (diabodies)" refers to a small antibody fragment having two antigen-binding sites, and the fragment contains V_H-V_L wherein the heavy chain variable region (V_H) is connected to the light chain variable region (V_L) in the same polypeptide chain. When a short linker is used between the two regions so that the two regions cannot be connected together in the same chain, these two regions form pairs with the constant regions in another chain to create two antigen-binding sites. The diabody is described in detail in, for example, European patent No. 404,097, WO 93/11.161 and Holliger P. et al. (Proc. Natl. Acad. Sci. USA 90: 6444-6448, 1993).

[0033] A single-chain antibody (hereafter also referred to as single-chain Fv or sFv) or sFv antibody fragment contains the V_H and V_L regions of an antibody, and these regions exist on a single polypeptide chain. Generally, an Fv polypeptide further contains a polypeptide linker between the V_H and V_L regions, and therefore an sFv can form a structure necessary for antigen binding. See, Pluckthun "The Pharmacology of Monoclonal Antibodies" Vol. 113 (Rosenburg and Moore eds. (Springer Verlag, New York) pp. 269-315, 1994) for the review of sFv.

[0034] A multispecific antibody is an antibody that has specificity to at least two different kinds of antigens. Although such a molecule usually binds to two antigens (i.e., a bispecific antibody), the "multispecific antibody" herein encompasses antibodies that has specificity to more than two antigens (for example, three antigens). The multispecific antibody can be a full-length antibody or fragments thereof (for example, $F(ab')_2$ bispecific antibody).

[0035] The phrase "humanized antibody" in the present invention is an antibody produced by genetic engineering. Specifically, it refers to an antibody characterized by a structure wherein a part of or the entire CDR of the hypervariable region is derived from that of a monoclonal antibody of a non-human mammal (mouse, rat, hamster, etc.), and the framework region of the variable region and constant region are those derived from human immunoglobulin. Herein, the CDR of a hypervariable region refers to the three regions (CDR1, CDR2 and CDR3) directly binding to an antigen in a complementary manner and that exist in the hypervariable region of the variable region of an antibody. Whereas, the framework region of a variable region refers to the relatively conserved four regions (framework regions; FR1, FR2, FR3 and FR4) which intervene between the three above-mentioned CDR regions. Specifically, the "humanized antibody" in the present invention refers to antibodies wherein all regions except a part or the entire CDR of the hypervariable region of a monoclonal antibody derived from a non-human mammal is replaced with a corresponding region of a human immunoglobulin.

[0036] Furthermore, a humanized antibody may contain residues that do not exist in either the recipient antibody or the introduced CDR or the framework sequence. Such alterations are performed to precisely optimize the capability of the antibody. Generally, all humanized antibodies essentially contain at least one, typically two variable regions. In the antibody, all or essentially all of the CDR regions correspond to the CDR of a non-human immunoglobulin, and all or essentially all of the FRs are derived from a human immunoglobulin variable region. Optimally, the humanized antibody further may contain typically at least a part of the constant region of a human immunoglobulin. More details can be found in Jones P. T. et al. (Nature 321: 522-525, 1986), Riechmann L. et al. (Nature 332: 323-327, 1988) and Presta et al. (Curr. Op. Struct. Biol. 2: 593-596, 1992).

[0037] The term "variable" in the antibody variable region indicates that a certain region in the variable region highly varies among antibodies, and that the region is responsible for the binding and specificity of respective antibodies to their specific antigens. The variable regions are concentrated in three areas called CDR or hypervariable region within the variable regions of light and heavy chains. There are at least two methods to determine the CDR: (1) a technique based on sequence variation among species (i.e., Kabat et al., "Sequence of Proteins of Immunological Interest" (National Institute of Health, Bethesda) 1987); and (2) a technique based on crystallographic research of antigen-antibody complex (Chothia C. et al., Nature 342: 877-883, 1989). The area more highly conserved in the variable region is called FR. The variable regions of natural heavy and light chains mainly have β-sheet structures and form three loop-like connections, and in some cases, contain four FRs connected by CDRs that form a β-sheet structure. The CDRs in each chain is maintained very closely to the CDRs on the other chain by FRs and plays a role in the formation of the antigen-binding site of an antibody (see, Kabat et al.). The constant region does not directly participate in the binding of the antibody to the antigen. However, it shows various

effector functions, such as participation of the antibody in antibody dependent cytotoxicity.

[0038] The constant region of a human immunoglobulin has a unique amino acid sequences for each isotype, such as IgG (IgG1, IgG2, IgG3 and IgG4), IgM, IgA, IgD and IgE. In the present invention, the constant region of the above-mentioned humanized antibody may be of any isotype. Preferably, the constant region of human IgG is used. Moreover, there is no limitation on the FR of the variable region derived from a human immunoglobulin.

[0039] The term "antigen" in the present specification encompasses both complete antigens having immunogenicity and incomplete antigens (including haptens) without immunogenicity. Antigens include substances such as proteins, polypeptides, polysaccharides, nucleic acids and lipids; however, they are not limited thereto. As immunogens for antibody production, soluble antigens or fragments thereof connected to other molecules may be used. In the interest of transmembrane molecules, such as receptors, fragments thereof (for example, extracellular regions of receptors) may be used as immunogens. Alternatively, cells expressing transmembrane molecules may be used as immunogens. Such cells may be natural cells (for example, tumor cell lines) or cells transfected by recombinant techniques to express the transmembrane molecules. Any form of antigen known to those skilled in the art can be used to produce antibodies.

[0040] Herein, the phrase "antigen-binding protein" refers to proteins that have the ability to bind to an antigen.

[0041] The phrase "immunoglobulin superfamily" in the present specification refers to proteins that have the structural characteristic wherein one or multiple domains homologous to the constant or variable domain of an immunoglobulin are contained. The immunoglobulin superfamily includes the immunoglobulin (H chain and L chain), T cell receptor (α chain, β chain, γ chain and δ chain), MHC class I molecule (α chain), β_2 microglobulin, MHC class II molecule (α chain and β chain), CD3 (γ chain, δ chain and ϵ chain), CD4, CD8 (α chain and β chain), CD2, CD28, LFA-3, ICAM-1, ICAM-2, VCAM-1, PECAM-1, F_c receptor II, poly Ig receptor, Thy-1, NCAM, myelin-associated glycoprotein (MAG), Po, carcinoembryonic antigen (CEA), PDGF receptor and so on.

[0042] The phrase "pharmaceutical agent" in the present specification refers to substances that are administered to animals for purposes such as treatment or prevention of diseases, injuries and such, or improvement of health conditions.

[0043] 1. Amino Acid Alternation for Protein Stabilization

[0044] The present invention provides a method for stabilizing a protein wherein an amino acid adjacent to an amino acid being deamidated in the protein is substituted with another amino acid. The protein to be stabilized according to the present invention is not restricted in any way. A suitable example of the protein includes antibodies. Humanized antibodies or human antibodies are preferred as the antibody from the aspect of medical use.

[0045] In addition to asparagine, glutamine is also known as an amino acid that is deamidated (Scotchler J. W. and Robinson A. B., Anal. Biochem. 59: 319-322, 1974). When comparing peptides of 5 amino acids, the half-life of glutamine is 96 to 3409 days compared to the half-life of asparagine being 6 to 507 days. Namely, the reaction rate of deamidation of glutamine is very slow compared with that of asparagine (Bischoff R. and Kolbe H. V. J., J. Chromatogr. B. 662: 261-278, 1994). Deamidation of glutamine has not been detected in antibody preparations (Harris R. J., Kabakoff B., Macchi F. D., Shen F. J., Kwong M., Andya J. D. et al., J. Chromatogr. B. 752: 233-245, 2001). However, the deamidation reaction is supposed to be enhanced in vivo than in pharmaceutical preparations (Robinson N. E. and Robinson A. B., Proc. Natl. Acad. Sci. USA 98: 12409-12413, 2001). Therefore, to develop an antibody preparation with a long in vivo half-life, suppression of deamidation of glutamine, in addition to asparagine is considered to be necessary. The amino acid to be deamidated preferably is asparagine.

[0046] Amino acids other than glycine can be also considered as the amino acid adjacent to an deamidated amino acid and that can be substituted in a protein (Robinson N. E. and Robinson A. B., Proc. Natl. Acad. Sci. USA 98: 4367-4372, 2001). However, glycine is particularly known to cause deamidation of asparagine. Thus, the amino acid that is located adjacent to an amino acid that is deamidated preferably is glycine.

[0047] Generally, an antibody is inactivated by amino acid substitution in the CDR. However, the present inventors revealed that the activity of an antibody is retained even after the substitution of an amino acid adjacent to asparagine in the CDR, and hence the stability of the antibody can be improved. Therefore, according to the present invention, an amino acid adjacent to an asparagine in the CDR is effectively substituted with another amino acid. Glycine is a suitable target as the amino acid adjacent to the asparagine. Particularly, glycine contained in the "Asn-Gly" sequence that is particularly easily deamidated is the most suitable target.

[0048] According to the present invention, in addition to the amino acid adjacent to the above-mentioned deamidated amino acid, one or more of other amino acids can also be altered unless the stability and biological activity of the protein is reduced. When the protein is an antibody, biological activity indicates its activity to specifically bind to antigen. A preferred amino acid alternation is a conservative substitution from the viewpoint to maintain the property of the protein.

[0049] The alteration of an amino acid of a protein can be performed by methods to recombine the gene sequence encoding the protein. Techniques generally known in the art can be used for gene recombination.

[0050] When the protein is an antibody, the alteration of amino acids can be performed as follows. For example, variant antibodies or mutants wherein one or more amino acid residues are altered in one or more of the hypervariable regions of the antibody can be prepared. In addition, one or more mutations (for example, substitution) can be introduced into the framework residues of the mammalian antibody to improve the binding affinity of the mutant antibody to its antigen. Exemplary framework residues that can be altered include portions that directly bind to antigens by noncovalent bonds (Amit A. G. et al., Science 233: 747-753, 1986), portions that affect and/or influence the structure of

the CDR (Chothia C. and Lesk A. M., J. Mol. Biol. 196: 901-917, 1987) and/or portions that are involved in the VL-VH interaction (European patent No. 239,400, B1). According to an embodiment, the binding affinity of an antibody to an antigen is enhanced by altering one or more of such framework residues.

[0051] One useful method for producing mutant antibodiesis "Alanine-Scanning Mutagenesis" (Cunningham B. C. and Wells J. A., Science 244: 1081-1085, 1989; Cunningham B. C. and Wells J. A., Proc. Natl. Acad. Sci. USA 84: 6434-6437, 1991). According to this method, one or more residues of the hypervariable region are substituted with alanine or polyalanine residues to change the interaction between the antigen and the corresponding amino acids. The residues of the hypervariable region that showed functional sensitivity to the substitution are further distinguished in more detail by introducing further or other mutation to the substitution site. Therefore, although the site to introduce an amino acid sequence mutation is determined beforehand, the type of mutation does not have to be determined beforehand.

[0052] The ala mutant produced by this method is screened for its biological activity. Depending on the desired characteristics obtained by the scanned residues, a similar substitution of other amino acids may also be performed. Alternatively, there is also a method wherein the altered amino acid residue is more systematically identified. According to this method, the hypervariable region residues within a species-specific antibody involved in the binding of a first mammalian species antigen and the hypervariable region residues involved in the binding of a homologous antigen of a second mammalian species can be identified. In order to achieve this, Alanine-scanning is performed for the hypervariable region residues of the species-specific antibody. In the scanning, the binding of each ala mutant to the first and second mammalian species antigen is tested in order to identify (1) the hypervariable region residues involved in the binding of the first mammalian species (for example, human) antigen and (2) the site involved in the binding of the second mammalian species (for example, non-human) antigen homolog. Preferably, residues that are apparently involved in the binding of the second mammalian species (for example, non-human mammalian) derived-antigen but not in the binding of the first mammalian species (for example, human) derived-antigen are candidates for alteration. In another embodiment, residues that are clearly involved in the binding of the first and second mammalian species derived-antigens are selected for alteration. The alteration includes deletion of the residues and insertion wherein one or more residues are linked to the target residues; however, generally, alteration refers to substitution of the residues with other amino acids.

[0053] A nucleic acid molecule encoding an amino acid sequence mutant may be prepared by various methods known in the art. Such methods include, but are not limited to, oligo nucleotide mediated mutation (or site-specific mutation), PCR mutation or cassette mutation of a previously produced mutated or a non-mutated version of a species-specific antibody. Suitable methods for producing mutants include site-specific mutation (see Kunkel T. A., Proc. Natl. Acad. Sci. USA 82: 488-492, 1985) and such. Generally, mutant antibodies having improved biological characteristics have at least 75%, preferably at least 80%, more preferably at least 85%, further more preferably at least 90% and most preferably at least 95% amino acid sequence homology or similarity with the amino acid sequence of the variable region of the heavy or light chain of the original antibody. Sequence homology or similarity in the present specification is defined as the rate of the amino acid residues which are homologous (i.e., the same residues) or similar (i.e., the amino acid residues of the same group based on the above-mentioned general side chain characteristic) to the residues in the species specific antibody of the candidate sequence after alignment of the sequence and introducing a gap as needed in order to obtain the maximum sequence homology.

[0054] Alternatively, a mutant antibody can be constructed by systematic mutations of the CDR in the heavy and light chains of an antibody. Preferable methods for constructing such a mutant antibody include methods utilizing affinity maturation using phage display (Hawkins R. E. et al, J. Mol. Biol. 226: 889-896, 1992; Lowman H. B. et al, Biochemistry 30: 10832-10838, 1991). Bacteriophage coat protein fusion (Smith G. P., Science 228:1315-1317, 1985; Scott J. K. and Smith G. P., Science 249: 386-390, 1990; Cwirla S. E. et al., Proc. Natl. Acad. Sci. USA 87: 6378-6382, 1990; Devlin J. J. et al., Science 249: 404-406, 1990; review by Wells and Lowman, Curr. Opin. Struct. Biol. 2:597, 1992; U.S. Pat. No. 5,223,409) is known as a useful method to relate a displayed phenotype protein or peptide with the genotype of the bacteriophage particle encoding it. Moreover, a method to display the F(ab) region of an antibody on the surface of a phage is also known in the art (McCafferty et al., Nature 348:552, 1990; Barbas et al., Proc. Natl. Acad. Sci. USA 88:7978, 1991; Garrard et al., Biotechnology 9:1373, 1991). Monovalent phage display comprises the step of displaying a group of protein variants as fusions with a coat protein of the bacteriophage yet that only one copy of the variant is displayed on a few phage particles (Bass et al, Proteins 8:309, 1990).

[0055] Affinity maturation or improvement of equilibrium of the binding affinity of various proteins has been performed hitherto by mutagenesis, monovalent phage display, functional analysis and addition of desirable mutations of, for example, human growth hormone (Lowman and Wells, J. Mol. Biol. 234: 564-578, 1993; U.S. Pat. No. 5,534,617) and antibody F(ab) region (Barbas et al., Proc. Natl. Acad. Sci. USA 91:3809, 1994; Yang et al., J. Mol. Biol. 254:392, 1995). A library of many protein variants (10^6 molecules) different at specific sequence sites can be prepared on the surface of bacteriophage particles that contain DNAs encoding specific protein variants. The displayed amino acid sequence can be predicted from DNA by several cycles of affinity purification using immobilized antigen followed by isolation of respective bacteriophage clones.

[0056] 2. Production of Polyclonal Antibodies

[0057] Polyclonal antibodies are preferably produced in non-human mammals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of related antigen and adjuvant. The related antigen may be bound to a protein that is immunogenic to the immunized species, for example, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin or soybean trypsin inhibitor, using bifunctional agents or inducers, for example, maleimidebenzoylsulfosuccinimide ester (binding via a cysteine residue), N-hydroxysuccinimide (via a lysine residue), glutaraldehyde, succinic

anhydride, thionylchloride or $R^1N{=}C{=}CR$ (wherein, R and R^1 are different alkyl groups).

[0058] For example, an animal is immunized against an antigen, an immunogenic conjugate or a derivative through multiple endermic injections of solution containing 100 μg or 5 μg of protein or conjugate (amount for a rabbit or a mouse, respectively) with 3 volumes of Freund's complete adjuvant. One month later, a booster is applied to the animal through subcutaneous injections of ⅕ to 1/10 volume of the original peptide or conjugate in Freund's complete adjuvant at several sites. Blood is collected from the animal after 7 to 14 days and serum is analyzed for antibody titer. Preferably, a conjugate of the same antigen but that is bound to a different protein and/or bound via a different cross-linking reagent is used as the booster. A conjugate can be also produced by protein fusion through recombinant cell culture. Moreover, in order to enhance immune response, agglutinins, such as alum, are preferably used. The selected mammalian antibody usually has a binding affinity strong enough to the antigen. The affinity of an antibody can be determined by saturation bonding, enzyme-linked immunosorbent assay (ELISA) and competitive analysis (for example, radioimmunoassay).

[0059] As a method of screening for desirable polyclonal antibodies, conventional cross-linking analysis described in "Antibodies, A Laboratory Manual" (Harlow and David Lane eds., Cold Spring Harbor Laboratory, 1988) can be performed. Alternatively, for example, epitope mapping (Champe et al., J. Biol. Chem. 270: 1388-1394, 1995) may be performed. Preferred methods for measuring the efficacy of a polypeptide or antibody include a method using the quantitation of the antibody binding affinity. Other embodiments include methods wherein one or more of the biological properties of an antibody are evaluated instead of the antibody binding affinity. These analytical methods are particularly useful in that they indicate the therapeutic efficacy of an antibody. Antibodies that show improved properties through such analysis have also generally, but not always, enhanced binding affinity.

[0060] 3. Production of Monoclonal Antibodies

[0061] A monoclonal antibody is an antibody that recognizes a single antigen site. Due to its uniform specificity, a monoclonal antibody is generally useful than a polyclonal antibody which contains antibodies recognizing many different antigen sites. A monoclonal antibody can be produced by the hybridoma method (Kohler et al., Nature 256:495, 1975), the recombinant DNA method (U.S. Pat. No. 4,816, 567), and so on.

[0062] According to the hybridoma method, a suitable host animal, such as mouse, hamster or rhesus monkey, is immunized similar as described above to produce antibodies that specifically bind to a protein used for immunization or to induce lymphocytes producing the antibodies. Alternatively, a lymphocyte may be immunized in vitro. Then, the lymphocyte is fused with a myeloma cell using suitable fusion agents, such as polyethylene glycol, to generate a hybridoma cell (Goding, "Monoclonal Antibodies: Principals and Practice", Academic Press, pp. 59-103, 1986). Preferably, the produced hybridoma cell is seeded and cultured on a proper culture media containing one or more substances that inhibit proliferation or growth of unfused parental myeloma cells. For example, when the parental myeloma cell lacks the hypoxantin guanine phosphoribosyl transferase enzyme (HGPRT or HPRT), the culture media for the hybridoma typically contains substances that inhibit the growth of HGRPT deficient cells, i.e., hypoxantin, aminopterin and thymidine (HAT culture media).

[0063] Preferred myeloma cells include those that can efficiently fuse, produce antibodies at a stable high level in selected antibody producing cells, and are sensitive to media such as HAT media. Among the myeloma cell lines, preferred myeloma cell lines include mouse myeloma cell lines, such as mouse tumor derived cells MOPC-21 and MPC-11 (obtained from Salk Institute Cell Distribution Center, San Diego, USA), and SP-2 and X63-Ag8-653 cells (obtained from the American Type Culture Collection, Rockville, USA). Human myeloma and mouse-human heteromycloma cell lines have also been used for the production of human monoclonal antibodies (Kozbar, J. Immunol. 133:3001, 1984; Brodeur et al., "Monoclonal Antibody Production Techniques and Application", Marcel Dekker Inc, New York, pp. 51-63, 1987).

[0064] Next, the production of monoclonal antibodies against an antigen in the culture media wherein the hybridoma cells had been cultured is analyzed. Preferably, the binding specificity of the monoclonal antibody produced from the hybridoma cells is measured by in vitro binding assay, such as immunoprecipitation, radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). After identifying the hybridoma cells that produce antibodies having the desired specificity, affinity and/or activity, clones are subcloned by limiting dilution method and cultured by standard protocols (Goding, "Monoclonal Antibodies: Principals an Practice", Academic Press, pp. 59-103, 1986). Culture media suitable for this purpose include, for example, DMEM and RPMI-1640. Furthermore, a hybridoma cell can also be grown as ascites tumor in an animal in vivo. Monoclonal antibodies secreted from a subclone are preferably purified from culture media, ascites or serum via conventional immunoglobulin purification methods, such as protein A-Sepharose, hydroxyapatite chromatography, gel electrophoresis, dialysis or affinity chromatography.

[0065] DNA encoding a monoclonal antibody can be easily isolated and sequenced by conventional methods, for example, using an oligo nucleotide probe specifically binding to genes encoding the heavy and light chains of the monoclonal antibody. Hybridoma cells are preferred starting materials for obtaining such DNAs. Once the DNA is isolated, it is inserted into an expression vector and transformed into a host cell, such as *E. coli* cell, simian COS cell, Chinese hamster ovary (CHO) cell or myeloma cell, that produce no immunoglobulin protein unless being transformed, and monoclonal antibody is produced from the recombinant host cell. In another embodiment, an antibody or an antibody fragment can be isolated from an antibody phage library prepared by the method described by McCafferty et al. (Nature 348: 552-554, 1990). Clackson et al. (Nature 352: 624-628, 1991) and Marks et al. (J. Mol. Biol. 222: 581-597, 1991) describe the isolation of mouse and human antibodies using phage libraries, respectively. The following references describe the production of high affinity (nM range) human antibody by chain shuffling (Marks et al, Bio/Technology 10: 779-783, 1992), and combinatorial infection and in vivo recombination for producing large phage libraries (Waterhouse et al, Nucl. Acids Res. 21:

2265-2266, 1993). These techniques can also be used to isolate monoclonal antibodies in place of conventional monoclonal antibody hybridoma techniques.

[0066] DNA can be also altered by, for example, substitution of corresponding mouse sequences with the coding sequences of the constant regions of human heavy and light chains (U.S. Pat. No. 4,816,567; Morrison et al, Proc. Natl. Acad. Sci. USA 81:6851, 1984), or by binding immunoglobulin polypeptides through covalent bonds. Typically, these non-immunoglobulin polypeptides are substituted with the constant region of an antibody or the variable region of the antibody antigen-binding site is substituted to construct a chimeric bispecific antibody that has an antigen-binding site specific for an antigen and another antigen-binding site specific for another antigen.

[0067] 4. Production of Antibody Fragments

[0068] Hitherto, antibody fragments have been produced by digesting natural antibody with proteases (Morimoto et al., J. Biochem. Biophys. Methods 24: 107-117, 1992; Brennan et al., Science 229:81, 1985). However, today, they can also be produced by recombinant techniques. For example, antibody fragments can also be isolated from the above-mentioned antibody phage library. Furthermore, $F(ab')_2$-SH fragments can be directly collected from a host cell such as *E. coli*, and chemically bound in the form of $F(ab')_2$ fragment (Carter, et al., Bio/Technology 10: 163-167, 1992). Moreover, in another method, $F(ab')_2$ fragment can also be directly isolated from recombinant host cell culture. The method for constructing single chain antibodies, fragments of single chain antibodies and such are well known in the art (for example, see, U.S. Pat. No. 4,946,778; U.S. Pat. No. 5,260,203; U.S. Pat. No. 5,091,513; U.S. Pat. No. 5,455,030; etc.).

[0069] 5. Production of Multispecific Antibodies

[0070] Methods for producing multispecific antibodies are known in the art. The production of a full-length bispecific antibody includes the step of co-expression of two immunoglobulin heavy-light chains having different specificity (Millstein et al., Nature 305: 537-539, 1983). The heavy and light chains of immunoglobulins are randomly combined, and therefore, the obtained multiple co-expressing hybridomas (quadroma) are a mixture of hybridomas each expressing a different antibody molecule. Thus, the hybridoma producing the correct bispecificity antibody has to be selected among them. The selection can be performed by methods such as affinity chromatography. Furthermore, according to another method, the variable region of an antibody having the desired binding specificity is fused to the constant region sequence of an immunoglobulin. The above-mentioned constant region sequence preferably contains at least a part of the hinge, CH2 and the CH3 regions of the heavy chain constant region of the immunoglobulin. Preferably, the CH1 region of the heavy chain required for the binding with the light chain is further included. DNA encoding the immunoglobulin heavy chain fusion is inserted into an expression vector to transform a proper host organism. If needed, DNA encoding the immunoglobulin light chain is also inserted into an expression vector different to that of the immunoglobulin heavy chain fusion to transform the host organism. There are cases where the antibody yield increases when the ratio of the chains is not identical. In such cases, it is more convenient to insert each of the genes into separate vectors since the expression ratio of each of the chains can be controlled. However, genes encoding plural chains can also be inserted into a vector.

[0071] According to a preferred embodiment, a bispecific antibody is desired wherein a heavy chain having a first binding specificity exists as an arm of the hybrid immunoglobulin and a heavy chain-light chain complex having another binding specificity exists as the other arm. Due to the existence of the light chain only on one of the arms, the bispecific antibody can be readily isolated from other immunoglobulins. Such a separation method is referred to in WO 94/04690. See, Suresh et al. (Methods in Enzymology 121:210, 1986) for further reference of methods for producing bispecific antibodies. A method wherein a pocket corresponding to a bulky side chain of a first antibody molecule is created in a multispecific antibody that comprises the antibody constant region CH3 (WO 96/27011) is also known as a method for decreasing homodimers to increase the ratio of heterodimers in the final product obtained from recombinant cell culture. According to the method, one of the antibody molecules is altered at one or more amino acids on the surface that binds to the other molecule to amino acids having a bulky side chain (e.g., tyrosine or tryptophan). Furthermore, amino acids with a bulky side chain in the corresponding portion of the other antibody molecule is altered to amino acids with a small side chain (e.g., alanine or threonine).

[0072] Bispecific antibodies include, for example, heteroconjugated antibodies wherein one antibody is bound to avidin and the other to biotin and such (U.S. Pat. No. 4,676,980, WO 91/00360, WO 92/00373, European patent No. 03089). Cross-linkers used for the production of such heteroconjugated antibodies are well known, and are mentioned, for example in U.S. Pat. No. 4,676,980.

[0073] Additionally, methods for producing bispecific antibodies from antibody fragments have been also reported. For example, bispecific antibodies can be produced utilizing chemical bonds. For example, first, $F(ab')_2$ fragments are produced and the fragments are reduced in the presence of dithiol complexing agent, sodium arsanilate, to prevent intramolecular disulfide formation. Next, the $F(ab')_2$ fragments are converted to thionitrobenzoate (TNB) derivatives. After re-reducing one of the $F(ab')_2$-TNB derivatives to a Fab'-thiol using mercaptoethanolamine, equivalent amounts of the $F(ab')_2$-TNB derivative and Fab'-thiol are mixed to produce a bispecific antibody.

[0074] Various methods have been reported to directly produce and isolate bispecific antibodies from recombinant cell culture. For example, a production method for bispecific antibodies using a leucine zipper has been reported (Kostelny et al., J. Immunol. 148:1547-1553, 1992). First, leucine zipper peptides of Fos and Jun proteins are connected to the Fab' sites of different antibodies by gene fusion, the homodimer antibodies are reduced at the hinge region to form monomers, followed by reoxidation to form a heterodimer antibody. Alternatively, a method to form two antigen-binding sites wherein pairs are formed between different complementary light chain variable regions (VL) and heavy chain variable regions (VH) by linking the VL and VH through a linker that is short enough to prevent binding between these two regions (Hollinger et al., Proc. Natl. Acad. Sci. USA 90: 6444-6448, 1993). Furthermore,

dimers utilizing single chain Fv (sFV) have also been reported (Gruger et al., J. Immunol. 152:5368, 1994). Moreover, trispecific (rather than bispecific) antibodies have also been reported (Tutt et al., J. Immunol. 147:60, 1991).

[0075] 6. Production of Humanized Antibodies

[0076] Humanized antibodies can be obtained via established general antibody production methods by immunizing a human antibody producing transgenic non-human mammal with an immunogen (antigen). Methods for producing human antibody producing non-human mammals, particularly human antibody producing transgenic mice, are known in the art (Nature Genetics 7: 13-21, 1994; Nature Genetics 15: 146-156, 1997; Published Japanese Translation of International Publication No. Hei 4-504365; Published Japanese Translation of International Publication No. Hei 7-509137; Nikkei Science 6: 40-50, 1995; WO 94/25585; Nature 368: 856-859, 1994; Published Japanese Translation of International Publication No. Hei 6-500233; etc.). Specifically, the human antibody producing transgenic non-human mammal can be produced by the following steps:

[0077] (1) producing a knockout non-human mammal wherein the endogenous immunoglobulin heavy chain gene of the animal is functionally inactivated via the substitution of at least a part of the endogenous immunoglobulin heavy chain locus of the non-human mammal with a drug resistance marker gene (for example, neomycin resistance gene) by homologous recombination;

[0078] (2) producing a knockout non-human mammal wherein the endogenous immunoglobulin light chain gene (particularly, the κ chain gene) of the animal is functionally inactivated via the substitution of at least a part of the endogenous immunoglobulin light chain locus of the non-human mammal with a drug resistance marker gene (for example, neomycin resistance gene) by homologous recombination;

[0079] (3) producing a transgenic non-human mammal wherein a desired region of the human immunoglobulin heavy chain locus has been integrated into the mouse chromosome using a vector represented by yeast artificial chromosome (YAC) vector and such that can transfer large genes;

[0080] (4) producing a transgenic non-human mammal wherein a desired region of the human immunoglobulin light chain (particularly, the κ chain) locus has been integrated into the mouse chromosome using a vector represented by YAC vector and such that can transfer large genes; and

[0081] (5) producing a transgenic non-human mammal wherein both the endogenous immunoglobulin heavy chain and light chain loci of the non-human mammal are functionally inactivated, yet desired regions of both the human immunoglobulin heavy chain and light chain are integrated into the non-mammalian chromosome by crossing the knockout non-human mammals and transgenic non-human mammals of above-mentioned (1) to (4) in an arbitrary order.

[0082] As mentioned above, an endogenous immunoglobulin locus of non-human mammals can be inactivated so that it inhibits reconstitution of the locus via the substitution of a proper region of the locus with an exogenous marker gene (for example, neomycin resistance gene, etc.) via homologous recombination. For inactivation using the homologous recombination, for example, a method called positive negative selection (PNS) can be used (Nikkei Science 5: 52-62, 1994). The functional inactivation of an immunoglobulin heavy chain locus can be attained by, for example, introducing a deficit into a part of the J or C region (for example, the Cμ region). On the other hand, the functional inactivation of an immunoglobulin light chain (for example, the κ chain) can be attained by, for example, introducing a deficit into a part of the J or C region, or a region comprising the area that spans over both the J and C regions.

[0083] A transgenic animal can be produced by standard methods (for example, "Saishin-Dobutsusaibou-Jikken manual (The latest animal cell experiment manual)", Chapter 7, LIC, pp. 361-408, 1990). Specifically, a hypoxantin-guanine phosphoribosyltransferase (HRPT) negative embryonic stem (ES) cell derived from normal non-human animal blastocyst is fused by the spheroplast fusion method with yeast that comprises a YAC vector inserted with a gene or a part thereof encoding the human immunoglobulin heavy chain or light chain locus and the HRPT gene. The ES cell wherein the exogenous gene has been integrated into the mouse endogenous gene is selected by HAT selection. Subsequently, the selected ES cell is microinjected into a fertilized egg (blastocyst) obtained from another normal non-human mammal (Proc. Natl. Acad. Sci. USA 77: 7380-7384, 1980; U.S. Pat. No. 4,873,191). A chimeric transgenic non-human mammal is born by transplanting the blastocyst into the uterus of another non-human mammal that acts as the surrogate mother. Heterotransgenic non-human mammals are obtained by crossing the chimeric animal with a normal non-human mammal. By crossing the heteroanimals among themselves, homotransgenic non-human mammals can be obtained according to Mendel's law.

[0084] A humanized antibody can also be obtained from the culture supernatant by culturing a recombinant human monoclonal antibody producing host that can be obtained via the transformation of the host with cDNAs encoding each of the heavy and light chains of such humanized antibody or preferably a vector containing the cDNAs by recombinant technique. Herein, such a host is a eukaryotic cell other than fertilized egg, preferably a mammalian cell, such as CHO cell, lymphocyte and myeloma cell.

[0085] The antigen-binding activity of an antibody stabilized by the method of the present invention is not particularly limited; however, it is preferred to have 70% or more, more preferably 80% or more and further preferably 90% or more of the activity possessed by the antibody before the amino acid substitution.

BRIEF DESCRIPTION OF THE DRAWINGS

[0086] **FIG. 1** depicts the amino acid sequences of humanized heavy chain version i and humanized light chain version b2 contained in the anti-human TF antibody described in WO 99/51743. A few asparagine residues (Asn28, Asn51 and Asn55) that may be deamidated are boxed.

[0087] **FIG. 2** depicts the cloning vector pCVIDEC-AHi integrated with the heavy chain variable region (AHi) of the

anti-human TF antibody. A: the entire pCVIDEC-AHi vector; and B: the NheI-SalI fragment of the heavy chain variable region.

[0088] **FIG. 3** depicts the anion exchange chromatogram of each of the anti-human TF mutant antibodies and the original anti-human TF antibody. A: 99D01; and B: original (native).

[0089] **FIG. 4** depicts the anion exchange chromatogram of each of the anti-human TF mutant antibodies and the original anti-human TF antibody. C: N28D; and D: N51D.

[0090] **FIG. 5** depicts the anion exchange chromatogram of each of the anti-human TF mutant antibodies and the original anti-human TF antibody. E: N54D; and F: N51D/N54D.

[0091] **FIG. 6** depicts the superposed anion exchange chromatograms of each of the anti-human TF mutant antibodies and the original anti-human TF antibody.

[0092] **FIG. 7** depicts the binding activity of each of the anti-human TF mutant antibodies and the original anti-human TF antibody.

[0093] **FIG. 8** depicts the neutralizing activity of each of the anti-human TF mutant antibodies and the original anti-human TF antibody.

[0094] **FIG. 9** depicts the cloning vector pCVIDEC-AHi integrated with the heavy chain variable region (AHi) of the anti-human TF antibody. The nucleotide sequences described in **FIG. 9** are shown in SEQ ID NOs: 27 and 28. A: the entire pCVIDEC-AHi vector; B: the XbaI-BalI fragment of the heavy chain variable region; and C: the XbaI-ApoI fragment of the heavy chain variable region.

[0095] **FIG. 10** depicts a graph comparing the binding activity of each of the anti-human TF mutant antibodies. The anti-human TF antibody concentration conversion values calculated based on the calibration curve plotted using the bulk anti-human TF antibody (Lot No. 00C01) are shown. Blank: 10% FCS-α-MEM culture medium; control: CHO cell culture supernatant; G to P: each of the anti-human TF antibody heavy chain Gly55 mutants.

[0096] **FIG. 11** depicts the binding activity of each of the anti-human TF mutant antibodies mutants.

[0097] **FIG. 12** depicts the elution profiles of each of the anti-human TF mutant antibodies and the bulk anti-human TF antibody (99D01).

[0098] **FIG. 13** depicts graphs showing the binding activity before and after the accelerated testing on each of the anti-human TF mutant antibodies and the bulk anti-human TF antibody (99D01), and the ratio compared with the initial value.

[0099] **FIG. 14** depicts the anion chromatograph and neutralizing activity before and after the accelerated testing on each of the anti-human TF mutant antibodies and the bulk anti-human TF antibody (99D01). A: 99D01 (G55G); B: G55L; C: G55I; D: G55F; E: G55E; and F: G55K.

[0100] **FIG. 15** depicts the anion chromatograph and neutralizing activity before and after the accelerated testing on each of the anti-human TF mutant antibodies and the bulk anti-human TF antibody (99D01). A: 99D01 (G55G); B: G55L; C: G55I; D: G55F; E: G55E; and F: G55K.

[0101] **FIG. 16** depicts the neutralizing activity before and after the accelerated testing on each of the anti-human TF mutant antibodies and the bulk anti-human TF antibody (99D01).

[0102] **FIG. 17** depicts the neutralizing activity before and after the accelerated testing on each of the anti-human TF mutant antibodies and the bulk anti-human TF antibody (99D01).

BEST MODE FOR CARRYING OUT THE INVENTION

[0103] Herein below, the present invention will be specifically described using Examples; however, it is not to be construed as being limited thereto:

EXAMPLE 1

Measurement of Binding and Neutralizing Activities of Anti-Human TF Antibody Asn54 Substitution Mutant with TF

[0104] Humanized antibody against human tissue factor (TF) described in WO 99/51743 is expected to suppress thrombus formation without suppressing the extrinsic blood coagulation reaction through the inhibition of the TF mediated Factor X activation in the intrinsic blood coagulation reaction. This anti-human TF antibody contains humanized heavy chain version i (SEQ ID NO: 25, **FIG. 1**) and humanized light chain version b2 (SEQ ID NO: 26, **FIG. 1**). The antibody comprises a few asparagine residues that may be deamidated: such as Asn51 and Asn54 in CDR2 of the heavy chain variable region, and Asn28 in FR1 of the heavy chain variable region. Particularly, Asn54 is contained in an Asn-Gly sequence, and thus is considered to be easily deamidated.

[0105] Pharmaceutical formulation of the antibody has not been established. Under destabilizing conditions of the antibody, the antibody binding activity to TF decreases in a solution pH-dependent manner and the increase of low pI molecular species has been observed. Due to the increase of degeneration upon stronger basification, the decrease in the binding activity and the increase of low pI molecular species are supposed to result from the deamidation of amino acids constituting the anti-human TF antibody. Furthermore, the deamidation is suggested to occur in the CDR region due to the co-observed decrease in the antigen binding activity.

[0106] Based on these findings, mutants (4 mutants including N51D mutant, N54D mutant, N51D/N54D double mutant and N28D mutant) wherein Asn51 and Asn54 in the CDR2 and Asn28 in the FR1 of the heavy chain variable region of anti-human TF antibody described in WO 99/51743 have been substituted with aspartic acid were prepared, and their binding activity and neutralizing activity to TF were measured.

[0107] The amino acid sequence of the antibody follows the sequence described by Kabat et al. (Kabat E. A., Wu T. T., Perry H. M., Gottesman K. S. and Foeller C., "Sequences of proteins of immunological interest. 5th ed.", US Dept. Health and Human Services, Bethesda, Md., 1991).

[0108] 1. Construction of Anti-Human TF Mutant Antibody Expression Vector

[0109] Cloning vector pCVIDEC-AHi (**FIG. 2A**) and anti-human TF antibody expression vector pN5KG4P-AHi-ALb2 both integrated with the heavy chain variable region (AHi) of the anti-human TF antibody were purified from dam$^-$/dcm$^-$*E. coli* SCS110.

[0110] Substitution of the codon encoding Asn with that of Asp was performed in pCVIDEC-AHi. Specifically, a fragment of about 30 bp containing the region that encodes each Asn was digested with restriction enzymes and replaced with a fragment prepared from a synthetic oligo DNA having base substitution (**FIG. 2B**). To alter Asn51 and Asn54, pCVIDE-AHi was digested with XbaI and BalI, and a fragment designed for one base pair substitution of the codon was integrated to alter either or both of Asn51 and Asn54 in the heavy chain variable region CDR2 of the anti-human TF antibody to Asp. To alter Asn28, pCVIDEC-AHi was digested with MroI and EcoT22I, and a fragment designed for one base pair substitution of the codon was integrated to substitute Asn28 to Asp in the heavy chain variable region FR1 of the anti-human TF antibody.

[0111] The sequence was confirmed at every step while constructing the expression vector. The target sequence was confirmed on the cloning vector, and the sequence was reconfirmed after replacing the fragment obtained by digestion with NheI and SalI with the heavy chain variable region of the anti-human TF antibody expression vector digested with NheI and SalI. *E. Coli* DH5α was transformed after confirming that the target sequence was obtained. Then, the four anti-human TF mutant antibody expression vectors, i.e., N51D mutant expression vector, N54D mutant expression vector, N51D/N54D double mutant expression vector and N28D mutant expression vector, were purified using the QIAGEN Maxi column.

[0112] 2. Transient Expression of Anti-Human TF Mutant Antibody in COS-7 Cell

[0113] Five vectors in total, i.e., the constructed expression vectors for each of the mutants and the original anti-human TF antibody, were transfected into COS-7 cells by the electroporation method and were transiently expressed. COS-7 cells were washed with D-PBS (−) and then resuspended in PBS to be about 0.3 to 1.0×10^7 cells/ml. The cell suspension was transferred into a 0.4 cm cuvette together with 10 μg of anti-human TF mutant antibody expression vector, and electroporation was conducted with the conditions of 1.5 kV and 25 μF. After leaving standing for 10 min, the cells were suspended in 30 ml of 10% FCS-DMEM. On the next day, dead cells were discarded together with the media and 50 ml of fresh 10% FCS-DMEM was added. The cells were cultured for 3 days and then the culture supernatant was collected.

[0114] 3. Measurement of Expression Level of Anti-Human TF Mutant Antibody

[0115] 3-1 Measurement of Expression Level by Direct ELISA

[0116] 100 μl each of the culture supernatant of the transfected COS-7 cells were seeded on a 96-well ELISA plate and immobilized over night. Similarly, 100μ each of anti-human TF antibody (Lot No. 00C01) serially diluted (1 to 1000 ng/ml) with DMEM was seeded and immobilized on a 96-well ELISA plate for plotting a calibration curve. After blocking with ELISA dilution buffer, HRP-labeled anti-IgG antibody was reacted and color was developed by TMB. The reaction was quenched with 2 M sulfuric acid and the absorbance at 450 nm was measured with ARVO-SX5. The amount of anti-human TF antibody in the culture supernatant was calculated from the value of the anti-human TF antibody (Lot No. 00C01) seeded for the calibration curve.

[0117] As shown in Table 1, direct ELISA confirmed concentration and total expression level of anti-TF antibody of about 65 to about 100 ng/ml and about 3 to about 5 μg, respectively.

TABLE 1

	Concentration (ng/ml)	Dosage (ml)	Total expression level (μg)
Original	98.710	50	4.9
N28D	84.535	50	4.2
N51D	75.634	50	3.8
N54D	77.956	50	3.9
N51D/N54D	68.387	50	3.4

[0118] 4. Purification of Each Anti-Human TF Mutant Antibody

[0119] Each mutant was purified from 50 ml of the recovered culture supernatant using affinity chromatography (Protein A) and anion exchange chromatography (Mono Q).

[0120] 4-1 Affinity Chromatography

[0121] Affinity chromatography was performed under the following conditions:

[0122] System: SMART System (Amersham Pharmacia Biotech)

[0123] Column: HiTrap Protein A HP (0.7 cmϕ×2.5 cm, 1 ml, Amersham Pharmacia Biotech)

[0124] Equilibrating Buffer: D-PBS (−)

[0125] Washing Buffer: 10 mM Sodium phosphate buffer (pH 7.4)

[0126] Elution Buffer: 50 mM Acetic acid (pH 2 to 3)

[0127] After adjusting the pH to 7.4 with 0.5 M sodium monophosphate solution, a sample was concentrated 5-fold with Centriprep-50 and loaded at a flow rate of 1 ml/min onto the column equilibrated with 10 ml (10 C.V.) equilibrating buffer. The column was washed with 5 ml (5 C.V.) washing buffer at a flow rate of 0.5 ml/min, eluted with 5 ml (5 C.V.) elution buffer, and then collected as ten fractions, each containing 0.5 ml solution. Four fractions containing the antibody were combined and neutralized to pH 6 to 7 with 0.1 ml of 1 M Tris base.

[0128] 4-2 Anion Exchange Chromatography

[0129] Next, anion exchange chromatography was performed under the following conditions:

[0130] System: SMART System (Amersham Pharmacia Biotech)

[0131] Column: Mono Q PC 1.6/5 (0.16 cmϕ×5 cm, 0.1 ml, Amersham Pharmacia Biotech)

[0132] Buffer A: 50 mM Tris-HCl (pH 8.0)

[0133] Buffer B: 50 mM Tris-HCl (pH 8.0)/0.5 M NaCl

[0134] Sample was prepared by adding 0.1 ml of 1 M Tris base to the Protein A elution fraction obtained by affinity chromatography to adjust the pH to 8 to 9. The sample was loaded onto the column at a flow rate of 200 μl/min, and then eluted by gradient elution using a gradient program of 0% B/5 min, 0 to 60% B/30 min, 60 to 100% B/10 min and 100% B/10 min, with a flow rate of 50 μl/min. The eluate was collected as 50 μl fractions, and 2 to 4 fractions containing the antibody were combined and subjected for activity measurement.

[0135] The affinity chromatography and anion exchange chromatography resulted in 0.5 to 1.0 μg of antibody. The anion exchange chromatogram of each mutant is shown in FIGS. **3** to **5** and the superposed chromatograms of the mutants are shown in **FIG. 6**. In addition, the amount and recovery rate of the proteins are shown in Table 2. The N54D mutant and N51D/N54D double mutant were obtained as almost a single peak. However, subpeak was observed for the original anti-human TF antibody, N51D mutant and N28D mutant. Particularly, N51D mutant showed 2 subpeaks, and that with high contents.

TABLE 2

	Peak No.	Initial protein amount (μg)	Concentration (ng/ml)	Dosage (ml)	Total protein amount (μg)	Recovery (%)
Original	1	4.9	6969.568	0.10	0.70	16.5
	2		734.883	0.15	0.11	
N28D	1	4.2	5436.713	0.15	0.82	20.7
	2		320.086	0.15	0.05	
N51D	1	3.8	2643.388	0.15	0.40	18.2
	2		2724.396	0.10	0.27	
	3		143.479	0.15	0.02	
N54D		3.9	2811.046	0.20	0.56	14.4
N51D/ N54D		3.4	5255.977	0.20	1.05	30.9

[0136] 5. Measurement of TF Binding Activity

[0137] TF binding activity was measured by competitive ELISA using biotinylated anti-human TF antibody. Each of the anti-human TF mutant antibodies was expressed in COS-7, and purified using protein A affinity chromatography and anion exchange chromatography to be used as samples. The subpeaks observed during anion exchange chromatography of the original anti-human TF antibody, N28D mutant and N51D mutant were used for the measurement. Lot No. 00C01 was used as the anti-human TF antibody standard.

[0138] shTF was adjusted to 20 nM with coating buffer (hereafter, indicated as CB), dispensed at 100 μl/well into a 96-well plate and incubated at 4° C. overnight. Each well was washed three times with rinse buffer (hereafter, indicated as RB), 200 μl dilution buffer (hereafter, indicated as DB) was added to each well. The plate was left standing at room temperature for 2 hours for blocking. After discarding DB, 100 μl sample diluted by 2-fold serial dilution with DB containing 10,000-fold diluted biotinylated anti-human TF antibody was added to each well, and the plate was left standing at room temperature for one hour. The plate was washed three times with RB, 100 μl ALP-streptavidin diluted 5,000-fold with DB was dispensed to each well, and left standing for 1 hour at room temperature. Each well was washed 5 times with RB and SIGMA104 adjusted with substrate buffer (hereafter, indicated as SB) to 1 mg/ml was dispensed to each well. Plates were left standing for 30 min at room temperature for color development and measured with a microplate reader at a wavelength of 405 nm and a control wavelength of 655 nm.

[0139] The assessment of binding activity was performed as follows: a straight-line as the standard was obtained by linear-regression of the concentration (logarithmic conversion value)-absorbance of the original anti-TF human antibody. The absorbance of each sample within the range of 62.5 to 500 ng/ml was converted to standard antibody concentration (Cc) using this standard straight-line. The added antibody concentration was subtracted from Cc (Ca) to obtain the sample concentration ratio to the standard antibody that shows the same binding activity as the binding activity.

[0140] The measurement results on the binding activities are shown in **FIG. 7** and Table 3. The binding activity of each mutant was lower than that of the original anti-human TF antibody. The binding activity of the mutant (N54D mutant) of Asn54 located in CDR2 yet mostly expected to undergo deamidation decreased to about 10% of the original anti-human TF antibody. The binding activity of the mutant (N54D mutant) of Asn51 located in CDR2 similar to Asn54 was about 50% of the original anti-human TF antibody, and the degree of decrease was smaller than the N54D mutant. The N51D/N54D double mutant, a mutant of both the amino acids Asn51 and Asn 54, had a further decreased binding activity than the N54D mutant. On the other hand, the binding activity of the mutant (N28D mutant) of Asn28 located in FR1 was about 94% of the original anti-human TF antibody showing only a slight decrease. From these findings, deamidation of Asn51 and Asn54 located in CDR2, particularly Asn54 was indicated to greatly reduce the binding activity.

[0141] Furthermore, the comparison of the binding activity of the subpeaks (peak 2) observed in the original anti-human TF antibody, N28D mutant and N51D mutant to that of the main peak (peak 1) revealed lower binding activity in all the subpeaks to the mainpeak.

TABLE 3

	Binding Activity	
	Peak 1	Peak 2
Native	100%	70.6%
N28D	93.9%	46.3%
N51D	49.2%	29.0%
N54D	9.2%	
N51D/N54D	7.0%	

[0142] 6. Measurement of TF Neutralizing Activity

[0143] TF neutralizing activity was measured using hTF (Thromborel S), Factor VIIa and Factor X. Similar to the measurement of the binding activity, each of the anti-human TF mutant antibodies was expressed in COS-7 cells and purified using Protein A affinity chromatography and anion exchange column chromatography. Lot No. 00C01 was used for the anti-human TF antibody standard.

[0144] Coagulation factor VIIa and Thromborel S were diluted with assay buffer (TBS (pH 7.49) containing 5 mM $CaCl_2$ and 0.1% BSA; hereafter, indicated as AB) to 0.1 PEU/ml and 120-fold (v/v), respectively. 60 μl of these mixtures were dispensed to each well of a plate and left standing for 60 min at room temperature. ABX wherein Factor X is diluted with AB to 0.25 PEU/ml was used to dilute the samples and 40 μl of sample diluted to the desired concentration was dispensed to each well of the plate. The plate was left standing for 30 min at room temperature, and the reaction was quenched by adding 10 μl/well of 500 mM EDTA. S-2222 mixture was prepared by mixing one volume of S-2222, a chromogenic substrate, solution with one volume of MilliQ H_2O and two volumes of 0.6 mg/ml hexamethylene bromide solution. Fifty μl/well of the S-2222 mixture was dispensed into the plate and left standing at room temperature. After 30 min, measurements were performed using a micro plate reader at a measurement wavelength of 405 nm and a control wavelength of 655 nm.

[0145] Measurement results on the neutralizing activity are shown in **FIG. 8** and Table 4. The concentration of each of the mutant was calculated using the anti-human TF antibody standard as a standard, and the neutralizing activity ratio compared to the anti-human TF antibody standard was obtained. The neutralizing activity ratio bases on the concentration of 250 ng/ml, at which concentration all samples could be measured. The original anti-human TF antibody and N28D mutant retained a neutralizing activity almost equivalent to the anti-human TF antibody standard. Thus, the deamidation of Asn28 located in FR was considered not to affect the decrease of neutralizing activity.

[0146] On the other hand, the neutralizing activity ratios of N51D and N54D mutants against the anti-human TF antibody standard decreased to 65.6% and 19.9%, respectively. Therefore, the deamidation of Asn51 and Asn54 located in CDR of the anti-human TF antibody was strongly suggested to cause the decrease of neutralizing activity.

TABLE 4

	Added concentration (ng/ml)	Calculated concentration (ng/ml)	Neutralizing activity ratio (%)
N28D	250	253	101
N51D	250	164	65.6
N54D	250	49.8	19.9
N51D/N54D	250	31.3	12.5
Native	250	248	99.1

[0147] From the results described above, the solution pH-dependent decrease in the binding activity to TF and increase in low pI molecular species of unformulated anti-human TF antibody under antibody destabilizing conditions were revealed to mainly result from the deamidation of Asn54 in the CDR2 region.

EXAMPLE 2

Measurement of TF Binding and Neutralizing Activities of Gly55 Substitution Mutant of Anti-Human TF Antibody

[0148] The anti-human TF antibody described in WO 99/51743 contains the humanized heavy chain version i (SEQ ID NO: 25, **FIG. 1**) and the humanized light chain version b2 (SEQ ID NO: 26, **FIG. 1**). Based on its amino acid sequence, mutants were prepared wherein the Gly55 in the heavy chain CDR2 that is considered as an important amino acid in the construction of the loop of CDR2 had been substituted with 19 other amino acids. Then, the binding activity of each mutant with TF was measured. Furthermore, the neutralizing activity and deamidation was observed for the mutants wherein Gly55 had been substituted with Ile, Leu, Phe, Glu and Lys.

[0149] The amino acid sequence of the antibody based on the sequence described by Kabat et al. (Kabat E. A., Wu T. T., Perry H. M., Gottesman K. S. and Foeller C., "Sequences of proteins of immunological interest. 5th ed.", US Dept. Health and Human Services, Bethesda, Md., 1991).

[0150] 1. Construction of Anti-Human TF Mutant Antibody Expression Vector

[0151] The cloning vector pCVIDEC-AHi (**FIG. 9A**) and the anti-human TF antibody expression vector pN5KG4P-AHi-ALb2 carrying the heavy chain variable region (AHi) of the anti-human TF antibody were isolated from *E. coli* SCS110 (dam^-/dcm^{-1}).

[0152] The substitution of the codon encoding Gly55 with a different amino acid was performed on the pCVIDEC-AHi. In this procedure, the substitution to 15 amino acids wherein the third codon can be fixed to "C" was performed as follows: digesting a fragment of about 30 bp comprising the coding region of Asn54-Gly55 at the unique sites XbaI and BalI of pCVIDEC-AHi, and integrating a fragment prepared using a synthetic oligo DNA wherein the 2 nucleotides at the 3'-end of the Gly55-coding codon has been randomized (**FIG. 9B**). The XbaI-BalI fragment was prepared by elongating the 3'-end with two nucleotides using Vent polymerase (NEB, Inc.) so that the 1st and 2nd nucleotides of the Gly55 codon in the CDR2 variable of the of the anti-human TF antibody heavy chain region become random nucleotide sequences, and then digesting with XbaI. This procedure was believed to enable production of 15 mutants with high codon usage in mammals via one operation. However, in fact, only 8 kinds of mutants were produced since optimal reaction conditions could not be found. Therefore, the remaining mutants were constructed using other restriction enzyme sites.

[0153] Mutants comprising substitution of an amino acid wherein the 3rd codon of Gly55 has to be converted, as well as those that could not be produced by the above-descried method were produced as follows: a vector wherein the EcoRI site of pCVIDEC-AHi is changed to HindIII site was constructed, digested at the unique sites ApoI and XbaI of pCVIDEC-AHi, and a fragment produced using synthetic oligo DNA was inserted. That is, apart from the XbaI and BalI sites, ApoI and XbaI sites were the possible sites that can be used as the restriction enzyme sites. However, ApoI also digests the ECORI sites in the vector. Therefore, the EcoRI site was first removed by changing it to a HindIII site. The ApoI-XbaI fragment was about 55 bp. Thus, a synthetic oligo DNA was prepared so that a total of about 16 bp overlap upstream and downstream of the nucleotide sequence of the codon encoding Gly 55 that is changed to other amino acids. After annealing, the fragment was elongated using Vent polymerase and digested with ApoI and XbaI (**FIG. 9C** and Table 5).

TABLE 5

EcoRI site deletion adapter G AATTC CTTAA G	Hind III AATTGGAAGCTTGC CCTTCGAACGTTAA
H-G56M primer-F	GAGTCTAGAATGGATTGGTGGGAATGATCCTGCGAATATGC
H-G56M primer-R	GAGAATTTCGGGTCATACATACTATGCATATTCGCAGGAT
H-G56M primer-F	GAGTCTAGAATGGATTGGTGGGAATGATCCTGCGAATAAGCAT
H-G56K primer-R	GAGAATTTCGGGTCATACATACTATGCTTATTCGCAGGAT
H-G56W primer-F	GAGTCTAGAATGGATTGGTGGGAATGATCCTGCGAATTGGCAT
H-G56W primer-R	GAGAATTTCGGGTCATACATACTATGCCAATTCGCAGGAT
H-G56Q primer-F	GAGTCTAGAATGGATTGGTGGGAATGATCCTGCGAATCAGCAT
H-G56Q primer-R	GAGAATTTCGGGTCATACATACTATGCTGATTCGCAGGAT
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H-G56E primer-R	GAGAATTTCGGGTCATACATACTATGCTCATTCGCAGGAT
H-G56F primer-F	GAGTCTAGAATGGATTGGTGGGAATGATCCTGCGAATTTCCAT
H-G56F primer-R	GAGAATTTCGGGTCATACATACTATGGAAATTCGCAGGAT
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H-G56P primer-R	GAGAATTTCGGGTCATACATACTATGGGGATTCGCAGGAT
H-G56C primer-F	GAGTCTAGAATGGATTGGTGGGAATGATCCTGCGAATTGCCAT
H-G56C primer-R	GAGAATTTCGGGTCATACATACTATGGCAATTCGCAGGAT

[0154] Primers for the construction of anti-human TF mutant antibodies using XbaI and ApoI sites are shown.

[0155] In addition, the nucleotide sequences indicated in Table 5 are shown in SEQ ID NOs: 1 to 24.

[0156] The sequences of the constructed 19 different anti-human TF mutant antibodies were confirmed in the cloning vector by a sequencer. Furthermore, the sequences were reconfirmed after constructing mutant expression vectors by replacing the heavy chain variable region obtained through NheI and SalI digestion with the heavy chain variable region digested from the anti-human TF antibody expression vector with NheI and SalI. After confirming that the target sequence was obtained, the anti-human TF mutant antibody expression vector was amplified using *E. coli* DH5α, purified using QIAGEN Maxi column and the sequence was confirmed. As a result, 19 different anti-human TF mutant antibody expression vectors were obtained.

[0157] 2. Transient Expression of Anti-Human TF Mutant Antibody in CHO Cells

[0158] A total of 20 expression vectors, i.e., the constructed anti-human antibody heavy chain Gly55 mutant expression vectors and Gly55 non-substituted antibody (Gly55Gly) expression vector, were transfected into CHO cells via the lipofection method for transient expression. A day before lipofection, the CHO (dhfr-) cells were cultured on 10% FCS-α-MEM in an atmosphere of 5% CO_2 at 37° C. The CHO cells were seeded at 1×10^5 cells/well on 12-well plates and cultured at 5% CO_2 at 37° C.

[0159] After adding 6 μl of FuGENE6 Transfection Reagent (Boehringer Mannheim GmbH) to 100 μl of Opti-MEM (GIBCO BRL) and leaving standing for 5 min, the mixture was added to a tube containing 1 μg of each of the anti-human TF antibody heavy chain Gly55 mutant expression vectors pN5KG4P-AHi-Alb2-G55X (X: 20 kinds of each amino acid). The tube was left standing for 20 min to form a FuGENE6/DNA complex. After discarding the media of the CHO cell seeded on the previous day, 2 ml/well of 10% FCS-α-MEM was newly added followed by the addition of each FuGENE6/DNA complex in triplicate.

[0160] The cells were cultured at 37° C. at 5% CO_2 for one day and then washed with PBS. Media were replaced by adding 3 ml/well of 10% FCS-α-MEM. After 7-day incubation at 5% CO_2 at 37° C., about 9 ml of culture supernatant containing each of the anti-human TF mutant antibody was transferred into a 15 ml tube, centrifuged at 1000 rpm for 5 min, and concentrated to 10-fold through ultrafiltration. The obtained culture supernatant was used as the anti-human TF antibody heavy chain Gly55Xaa mutant sample.

[0161] 3. Measurement of TF Binding Activity

[0162] Human IgG content in the anti-human TF antibody heavy chain Gly55Xaa mutant sample of was measured to adjust the IgG concentration of each sample to 100 ng/ml.

[0163] TF binding activity was measured by competitive ELISA using biotinylated anti-human TF antibody. shTF was adjusted to 20 nM with CB, dispensed at 100 μl/well into a 96-well plate, and was left standing at 4° C. overnight. The plate was washed three times with RB, 200 μl/well of DB was dispensed thereto, and then left standing for blocking at room temperature for 2 hours. After removing DB, 100 μl/well of standard and samples diluted by 2-fold serial dilution with DB containing biotinylated anti-human TF antibody (diluted 10,000-fold at final concentration) were added. The plates were left standing at room temperature for 0.1 hour. After washing 3 times with RB, 100 μl/well of ALP-streptavidin, diluted 8,000-fold with DB, was added and left standing at room temperature for one hour. SIGMA104 adjusted to 1 mg/ml with SB was added after washing 3 times with RB, and left standing for about 20 min at room temperature to develop colors and measure with a microplate reader at a wavelength of 405 nm and a control wavelength of 655 nm.

[0164] The binding activity was compared by determining the concentration that showed 50% activity according to the following procedure: the absorbance at each measured point was converted to percentage (%) by taking the absorbance of sample (−) and biotinylated antibody (+) as 100%. A linear regression equation of "concentration (logarithmic conversion value)−absorbance (%)" was obtained based on two points which sandwich the 50% value of each sample. Then, the concentration giving 50% absorbance was calculated to calculate the binding activity of each sample from Equation 1.

binding activity=(50% activity concentration of the standard antibody)/(50% activity concentration of sample)×100. Equation 1

[0165] The anti-human TF antibody concentration conversion value that was calculated based on the calibration curve produced using bulk anti-human TF antibody (Lot No. 00C01) is shown in **FIG. 10**. The Gly55 non-substituted antibody (Gly55Gly) expressed in CHO cells retained a TF binding activity almost equivalent to the bulk anti-human TF antibody. Decrease in the binding activity was observed in the Gly55 mutants, Gly55Val, Gly55Ile and Gly55Pro.

[0166] The following assay was performed to examine the TF binding activity of the anti-human TF antibody heavy chain Gly55 mutants in detail. Specifically, the TF binding activity was measured by competitive ELISA method using the anti-human TF mutant antibodies by changing the amount of added sample within the range of 25 to 200 ng/ml. Measurements on Gly55Asn and Gly55Asp were not performed due to the lack of sample amount.

[0167] The measurement results are shown in **FIG. 11**. Among the examined 18 anti-human TF antibody heavy chain Gly55 mutants, the TF binding activity of Gly55Val, Gly55Ile and Gly55Pro were significantly decreased compared with the bulk anti-human TF antibody (Lot No. 00C01) and Gly55 non-substituted antibody (Gly55Gly). However, no significant difference in the TF binding activity could be observed for the other 15 mutants. Thus, the TF binding activity was supposed to be maintained even after changing the Gly55 with another amino acid.

[0168] 4. Measurement of TF Neutralizing Activity

[0169] Coagulation factor VIIa and Thromborel S were diluted with AB to 0.1 PEU/ml and 120-fold (v/v), respectively. Sixty μl/well of a mixture thereof was dispensed to a plate and left standing at room temperature for 60 min. Twenty μl/well of sample diluted by 2-fold serial dilution with 10 mM phosphate buffer was dispensed followed by 20 μl/well coagulation factor (Factor X) solution diluted to 0.5 PEU/ml with AB (supplemented with $CaCl_2$ solution to a $CaCl_2$ concentration of 10 mM). The plate was left standing at room temperature for 30 min and then the reaction was quenched by adding 10 μl/well of 500 mM EDTA. Fifty μl/well of a solution of test-team chromogenic substrate S-2222 solution and polybrene solution mixed at 1:1 was dispensed and left standing at room temperature. After 30 min, measurements were taken by a microplate reader at a measurement wavelength of 405 nm and a control wavelength of 655 nm.

[0170] The neutralizing activity was compared by determining the concentration showing 50% activity according to the following procedure: the absorbance at each measured point was converted to percentage (%) by taking the absorbance of sample (−) and coagulation factor X (+) as 100%, and sample (−) and coagulation factor X (−) as 0%. A linear regression equation of "concentration (logarithmic conversion value)−absorbance (%)" was obtained based on two points that sandwich the 50% value of each sample. Then, the concentration giving 50% absorbance was calculated to calculate the neutralizing activity of each sample from Equation 2.

Neutralizing activity (*IC*50)=(50% activity concentration of the standard antibody)/(50% activity concentration of sample)×100. Equation 2

[0171] 5. Construction of Stable Expression System of Anti-Human TF Mutant Antibodies Using CHO Cells

[0172] Five kinds of mutants, Gly55Leu, Gly55Phe, Gly55Glu, Gly55Lys and Gly55Ile, wherein the Gly55 is substituted to Leu (aliphatic amino acid), Phe (aromatic amino acid), Glu (acidic amino acid), Lys (basic amino acid) and Ile (branched-chain aliphatic amino acid), respectively, were produced in sufficient quantity to compare the activity of the anti-human TF mutant antibodies by constructing stable expression cell lines.

[0173] 5-1 Gene Transfer into CHO Cells

[0174] CHO (dhfr-) cells were washed with PBS and then resuspended in PBS to about 1×10^7 cells/ml. The cells were

transferred into a 0.4 cm cuvette together with 10 μg of the expression vector of the anti-human TF antibody heavy chain Gly55 mutant, pN5KG4P-AHi-Alb2-G55X. Electroporation was performed at 1.5 kV with 25 μF. After leaving standing for 10 min, the cells were suspended in 200 ml of 10% FCS-α-MEM nucleic acid (−) media. Two hundred μl/well of the suspension was seeded and cultured on ten 96-well plates.

[0175] 5-2 Selection of Transfected Cells

[0176] The amount of expressed antibody in wells wherein cell growth could be observed during the 96-well plate culture was compared by hIgG ELISA. Cells that showed high hIgG expression were subcultured from 10 wells each at a total of 70 wells into 12 well plates and cultured in 10% FCS-α-MEM nucleic acid (−) media. The expression amount of anti-human TF mutant antibody was measured by hIgG ELISA at the time when the cells had acclimatized to the 10% FCS-α-MEM nucleic acid (−) media and showed satisfactory growth. Four wells were selected for each mutant and subcultured into a 50 ml flask. Antibody production was enhanced by replacing the media with 10% FCS-α-MEM nucleic acid (−) containing 10 nM MTX.

[0177] 5-3 Production of Anti-Human TF Mutant Antibody by Large-Scale Culture Using Serum Free Media

[0178] Among the anti-human TF mutant antibody clones, one clone each for each mutant having a high hIgG expression level was selected and cultured in six 175 cm^2 flasks using media containing 10 nM MTX. The media were replaced with 150 ml CHO-S-SFM II serum free media after reaching subconfluence and incubated for 7 days. The culture supernatant was collected, treated with 0.22 μm filter, and stored at −80° C. until purification.

[0179] 5-4 Measurement of Expression Level of Anti-Human TF Mutant Antibody by Sandwich ELISA

[0180] One hundred μl/well of anti-human IgG (γ) antibody was dispensed into a 96-well plate and left standing at 4° C. overnight. After washing 3 times with RB, 200 μl/well of DB was dispensed and left standing at room temperature for 2 hours for blocking.

[0181] After discarding DB, 100 μl/well of the standard and sample that was properly diluted with DB or media used to recover the antibody from the anti-human TF mutant antibody producing cells was added, and left standing at room temperature for 2 hours. After washing three times with RB, 100 μl/well of HRP-labeled anti-human IgG antibody diluted 10,000-fold with DB was dispensed and left standing at room temperature for 1 hour. After washing 10 times with RB, 100 μl/well of chromogenic reagent was dispensed and left standing at room temperature for about 10 min. Color reaction was quenched by the addition of 50 μl/well of 2 N sulfuric acid to measure the absorbance with a microplate reader at a measurement wavelength of 450 nm and a control wavelength of 655 nm.

[0182] Consequently, several milligrams of each of the anti-human TF mutant antibodies, except Gly55Gly, were obtained (Table 6).

TABLE 6

Version	G55G	G55F	G55L	G55E	G55K	G55I	99D01
Clone No.	196	41	96	23	237	127	
α-MEM N (−) (ng/ml)	29	64	9	59	600	110	
α-MEM 10 nM MTX (ng/ml)	50	836	3451	6143	423	369	
CHO-SFM-II (large-Scale: 900 ml) (μg/ml)	0.24	15.4	41.7	50	11.5	5.8	
Purified (after buffer exchange: μg/ml, total 7 ml)	—	319	379	624	180	153 (4 ml)	1556

[0183] 6. Purification of Each Anti-Human TF Antibody Mutant

[0184] Each mutant was purified from the supernatant of the large scale culture containing each of the mutants using a HiTrap rProtein A FF column and a HiTrap Q Sepharose HP column.

[0185] 6-1 Affinity Chromatography for Purification

[0186] Affinity chromatography was performed in a refrigerated room under the following conditions:

[0187] System: FPLC System

[0188] Column: HiTrap rProtein A FF (1.6 cmϕ×2.5 cm, 5 ml)

[0189] Equilibrating buffer: D-PBS (−)

[0190] Washing buffer: 10 mM Sodium phosphate buffer (pH 7.4)

[0191] Elution buffer: 50 mM Acetic acid (pH 2 to 3)

[0192] Samples were loaded onto the column after adjusting the pH to 7.4 with 0.5 M disodium phosphate solution. Fifty ml of washing buffer was used to dilute 1.5 ml (16.5 mg) of the anti-human TF antibody standard. Elution was performed with 25 ml (5 C.V.) elution buffer at a flow rate of 5 ml/min and the pH was neutralized to 6 to 7 with 1.25 ml of 1 M Tris base.

[0193] 6-2 Anion Exchange Chromatography for Purification

[0194] Next, anion exchange chromatography was conducted in a refrigerated room under the following conditions:

[0195] System: FPLC System

[0196] Column: HiTrap Q Sepharose HP (0.7 cmϕ×2.5 cm, 1 ml)

[0197] Buffer A: 50 mM Tris-HCl (pH 8.0, 4° C.)

[0198] Buffer B: 50 mM Tris-HCl (pH 8.0, 4° C.)/1 M NaCl

[0199] Sample was prepared by adjusting the pH of the Protein A elution fraction obtained via affinity chromatography to 8 to 9 through the addition of 1.25 ml of 1 M Tris base. Elution steps of 0 mm NaCl (5 C.V.), 250 mM NaCl (5+5 C.V.) and 1 M NaCl (100 C.V.) at a flow rate of 1 ml/min were performed and the first half 5 C.V. (5 ml) of the 250 mM NaCl step was collected.

[0200] Five hundred μg or more of each of the anti-human TF mutant antibodies, except Gly55Gly, was obtained (Table 5). Gly55Gly was not obtained. Therefore, bulk anti-human TF antibody (Lot No. 99D01) was purified according to a similar procedure to use it for comparison with anti-human TF antibody (Table 5).

[0201] 7. Anion Exchange Chromatography for Analysis

[0202] Sample was analyzed by anion exchange chromatography at room temperature under the following conditions:

[0203] System: SMART System

[0204] Column: MonoQ PC 1.6/5 (0.16 cmϕ×5 cm, 0.1 ml)

[0205] Buffer A: 50 mM Tris-HCl (pH 8.0, 20° C.)

[0206] Buffer B: 50 mM Tris-HCl (pH 8.0, 20° C.)/500 mM NaCl

[0207] Gradient elution with a gradient program of 0% B/5 min, 0-60% B/30 min, 60 to 100% B/10 min and 100% B/10 min at a flow rate of 50 μl/min was performed. Two μg of sample (calculated by UV conversion) was diluted 3 to 50 times with 50 μl of buffer A and 25 μl thereof was analyzed.

[0208] The analysis by anion exchange chromatography of purified bulk anti-human TF antibody (99D01) and each of the anti-human TF mutant antibody revealed almost a single peak, although with a change in elution time depending on the introduced amino acid mutation (**FIG. 12**).

[0209] 8. Suppression of Anti-Human TF Antibody Deamidation by Amino Acid Mutation

[0210] In order to examine the deamidation reaction, accelerated testing was performed under heated condition using a neutral pH buffer wherein deamidation easily occurs.

[0211] 8-1 Replacement of Buffer

[0212] Replacement of sample buffer with 20 mM sodium phosphate buffer/150 mM sodium chloride (pH 7.5) buffer using a PD-10 desalting column was performed. After equilibrating the column, 2.5 ml sample was loaded onto two columns and eluted with 3.5 ml buffer.

[0213] 8-2 Sample Preparation for Accelerated Testing

[0214] Each sample of the anti-human TF mutant antibodies was diluted to 100 μg/ml based on the value quantitated by hIgG ELISA. Buffer containing 20 mM sodium phosphate buffer/150 mM NaCl (pH 7.5) was used. After passing through a 0.22 μm filter, 1 ml of each sample was dispensed into a 5 ml vial.

[0215] 8-3 Accelerated Testing

[0216] Accelerated testing on the purified bulk anti-human TF antibody (99D01) and anti-human TF mutant antibodies was performed for four weeks at 40° C. in 20 mM sodium phosphate buffer/150 mM NaCl (pH 7.5) solution. A portion was sampled at each point of 0, 1, 2 and 4 weeks, and its activity was analyzed through the comparison of TF binding activity and TF neutralizing activity. Deamidation at each point was analyzed using analytical anion exchange chromatography.

[0217] The value (Table 7) obtained by requantitation using the monomer fraction of GPC as an indicator was used for the comparison of activity. Specifically, quantitation of antibody was performed at room temperature under following conditions:

[0218] System: Waters (600S Controller, 616 Pump, 486 Tunable absorbance detector, 717 Plus Autosampler)

[0219] Column: TSK gel G3000SWXL (0.78 cmϕ× 30 cm, guard column 0.6 cmϕ×4 cm)

[0220] Buffer: 50 mM Sodium phosphate/300 mM NaCl (pH 7.0)

[0221] Analysis was performed at a flow rate of 0.5 ml/min using 100 μl (equivalent to 10 μg) of accelerated material as a sample.

TABLE 7

	Initial value	1 week	2 weeks	4 weeks
99D01	116.1	115.2	116.5	112.4
G55L	116.5	113.8	115.6	113.7
G55I	102.7	99.6	98.4	94.5
G55F	118.3	115.3	114.8	111.8
G55E	110.8	110.2	110.7	109.8
G55K	135.2	134.9	136.0	130.9

[0222] Similar to transiently expressed anti-human TF mutant antibodies, the result showed that the TF binding activity of Gly55Ile before the accelerated testing was about 26% of the bulk anti-human TF antibody (Lot No. 00C01). Namely, the activity was low and significantly reduced compared with 99D01 (**FIG. 13A**). Almost an equivalent activity to 99D01 was retained by Gly55Leu, Gly55Glu, Gly55Phe and Gly55Lys (**FIG. 13A**). After 4 weeks of accelerated testing, the anti-human TF mutant antibodies retained 80% or more of its activity before the accelerated testing, whereas the activity of 99D01 decreased to about 60% of its activity before the accelerated testing (**FIG. 13B**).

[0223] The analysis of deamidation using analytical anion exchange chromatography indicated significant increase in a peak considered to correspond to the deamidated molecule in 99D01 but almost none in the anti-human TF mutant antibodies (**FIG. 14**). In the interest of the changes in the TF neutralizing activity over time, 99D01 showed a relatively large reduction in activity (**FIG. 15**).

[0224] From these results, deamidation of Asn55 was determined to be suppressed by the substitution of Gly55, and amino acid substitution Gly55Leu and Gly55Phe were suggested suitable for suppressing deamidation.

[0225] 9. TF Neutralizing Activity Before and After Accelerated Testing

[0226] TF neutralizing activities of 99D01 and each of the anti-human TF mutant antibodies are shown in **FIG. 16**. Although Gly55Glu and Gly55Ile showed low activities of

about 41% and about 13%, respectively, the other 3 anti-human TF mutant antibodies had activities between 56 to 74%, i.e., nearly the same as 99D01 (66%).

[0227] The IC50 value compared to the initial value of each sample was calculated from **FIG. 15** in order to examine the amount of activity decrease over time of 99D01 and each of the anti-human TF mutant antibodies in the accelerated testing (**FIG. 17**). Since the IC50 value after the accelerated testing at 40° C. for 4 weeks could not be calculated for the Gly55Ile sample, the results up to 2 weeks are indicated for this sample. The TF neutralizing activity of 99D01 after the accelerated testing at 40° C. for 4 weeks decreased to about 40% of the initial value. On the other hand, each of the mutants wherein Gly55 was substituted with another amino acid maintained a TF neutralizing activity of 50 to 70% of the initial value even after the accelerated testing at 40° C. for 4 weeks.

[0228] These results indicate that substitution of a glycine that is located adjacent to an asparagine in an antibody with another amino acid does not decrease the antibody activity, yet it suppresses instability due to deamidation.

Industrial Applicability

[0229] The present inventors found that substitution of glycine that is located adjacent to asparagine with another amino acid does not influence the antibody activity. The present invention can be applied to produce antibodies showing low activity decrease, and thus, to obtain antibodies that can be used as pharmaceutical agents that are required to be stable for a long time. Furthermore, the present invention can also be applied to proteins other than antibodies, and are expected to achieve suppression of deamidation without affecting the protein activity.

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<223> OTHER INFORMATION: Description of Artificial Sequence:an artificially synthesized primer sequence

<400> SEQUENCE: 24

gagaatttcg ggtcatacat actatggcaa ttcgcaggat 40

<210> SEQ ID NO 25
<211> LENGTH: 444
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 25

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
1 5 10 15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
20 25 30

Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
35 40 45

Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
50 55 60

Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Val Phe
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
100 105 110

Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
115 120 125

Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala Ala Leu Gly Cys
130 135 140

Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser
145 150 155 160

Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser
165 170 175

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Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser
            180                 185                 190

Leu Gly Thr Lys Thr Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn
        195                 200                 205

Thr Lys Val Asp Lys Arg Val Glu Ser Lys Tyr Gly Pro Pro Cys Pro
    210                 215                 220

Pro Cys Pro Ala Pro Glu Phe Leu Gly Gly Pro Ser Val Phe Leu Phe
225                 230                 235                 240

Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val
                245                 250                 255

Thr Cys Val Val Val Asp Val Ser Gln Glu Asp Pro Glu Val Gln Phe
            260                 265                 270

Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro
        275                 280                 285

Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr
    290                 295                 300

Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
305                 310                 315                 320

Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala
                325                 330                 335

Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln
            340                 345                 350

Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly
        355                 360                 365

Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
    370                 375                 380

Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser
385                 390                 395                 400

Phe Phe Leu Tyr Ser Arg Leu Thr Val Asp Lys Ser Arg Trp Gln Glu
                405                 410                 415

Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His
            420                 425                 430

Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu Gly Lys
        435                 440


<210> SEQ ID NO 26
<211> LENGTH: 214
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 26

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
  1               5                  10                  15

Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Lys Ser Phe
             20                  25                  30

Leu Ser Trp Tyr Gln Gln Lys Pro Glu Lys Ala Pro Lys Ser Leu Ile
         35                  40                  45

Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
     50                  55                  60

Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro
 65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His Gly Glu Ser Pro Tyr
                 85                  90                  95
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Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
            100                 105                 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
        115                 120                 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
    130                 135                 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145                 150                 155                 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
                165                 170                 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
            180                 185                 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
        195                 200                 205

Phe Asn Arg Gly Glu Cys
    210


<210> SEQ ID NO 27
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:an
      artificially synthesized sequence

<400> SEQUENCE: 27

gagtctagaa tggattggtg ggaatgatcc tgcgaat                              37


<210> SEQ ID NO 28
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:an
      artificially synthesized sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(2)
<223> OTHER INFORMATION: n = g, a, c, or t

<400> SEQUENCE: 28

nnattcgcag gatcattccc accaatccat tctagactc                            39
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1. A method for stabilizing a protein, which comprises the step of substituting an amino acid that is located adjacent to an amino acid being deamidated with another amino acid.

2. The method for stabilizing a protein of claim 1, wherein the amino acid being deamidated is asparagine.

3. The method for stabilizing a protein of claim 1, wherein the amino acid that is located adjacent to the C-terminal side of the amino acid being deamidated is glycine.

4. The method for stabilizing a protein of any one of claims 1 to 3, wherein the protein is an antibody.

5. The method for stabilizing a protein of claim 4, wherein the antibody is humanized antibody.

6. The method for stabilizing a protein of claim 4 or **5**, wherein the amino acid being deamidated exists in the complementary determining region (CDR).

7. The method for stabilizing a protein of claim 6, wherein the complementary determining region (CDR) is CDR2.

8. The method for stabilizing a protein of any one of claims 1 to 3, wherein the protein is an antigen binding protein.

9. The method for stabilizing a protein of any one of claims 1 to 3, wherein the protein belongs to the immunoglobulin superfamily.

10. The method for stabilizing a protein of any one of claims 1 to 3, wherein the protein is a pharmaceutical agent.

11. A protein stabilized by the method of any one of claims 1 to 10.

12. The stabilized protein of claim 11 whose antigen binding activity is 70% or more of the activity before the amino acid substitution.

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