COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

# DECLARATION IN SUPPORT OF CONVENTION OR NON-CONVENTION APPLICATION FOR A PATENT

In support of the Application made for a patent for an invention

Insert title of invention.

entitled: "A method for determining chondrocalcin"

Insert full name(s) and address(es) of declarant(s) being the applicant(a) or person(s) authorized to sign on behalf of an applicant company.

I
~~We~~ Kazuo Ogata of
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Cross out whichever of paragraphs 1(a) or 1(b) does not apply

1(a) relates to application made by individual(s)

1(b) relates to application made by company; insert name of applicant company.

do solemnly and sincerely declare as follows:-

1. (a) ~~Kann We are the applicants for the patent~~

or (b) I am authorized by

Teijin Limited

the applicant..... for the patent to make this declaration on ~~their~~ its behalf.

Cross out whichever of paragraphs 2(a) or 2(b) does not apply

2(a) relates to application made by inventor(s)

2(b) relates to application made by company(s) or person(s) who are not inventor(s); insert full name(s) and address(es) of inventors.

2. (a) ~~Kann We are the actual inventor of the invention~~

or (b)
1. Kimihiko MATSUZAWA
2. Kazuhiko ITOU
3. Hitomi HONDA
4. Ryoichi HASEGAWA
5. Naomi OKAMOTO
6. Kenji HOSODA
7. Masayuki SHINMEI
8. Shigeo MATSUYAMA

See over page for addresses

~~is~~ are the actual inventor~~s~~..... of the invention and the facts upon which the applicant..... ~~is~~ are entitled to make the application are as follows:-

State manner in which applicant(s) derive title from inventor(s)

The actual inventors assigned the invention to the said applicant.

Cross out paragraphs 3 and 4 for non-convention applications. For convention applications, insert basic country(s) followed by date(s) and basic applicant(s).

3. The basic application~~s~~..... as defined by Section 141 of the Act ~~was~~ were made

in Japan ..... on the 10 May 1988

by Teijin Limited

in Japan ..... on the 9 August 1988

by Teijin Limited

in Japan ..... on the 10 August 1988

by Teijin Limited

4. The basic application~~s~~..... referred to in paragraph 3 of this Declaration ~~was~~ were the first application~~s~~..... made in a Convention country in respect of the invention the subject of the application.

Insert place and date of signature.

Declared at Osaka this 26th day of December 1989

Signature of declarant(s) (no attestation required)

Teijin Limited

Kazuo Ogata

Kazuo Ogata, Senior Executive

Note: Initial all alterations.

DAVIES & COLLISON, MELBOURNE and CANBERRA

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(57) Claim

1. A method for determining chondrocalcin by a sandwich immunoassay using an anti-chondrocalcin antibody immobilised to an insolubie support and an enzyme(s)-labelled anti-chondrocalcin antibody, the insoluble support comprising mirror-finished beads having Ra less than 1.5 μm, wherein a body fluid of mammalian is used as a specimen and the chondrocalcin of mammalian is determined utilising an immunoreaction in solution and a protein of 16,000 to 50,000 molecular weight and 1.0 to 5.0 isoelectric point or a mixture containing said protein is allowed to exist so that the final concentration of said protein in the mixture is adjusted to 0.02-0.9% by weight in the immunoreaction in solution.

8. A chondrocalcin-determining reagent for measuring immunologically the chondrocalcin in a body fluid of mammalian as a specimen, comprising an anti-chondrocalcin antibody immobilised on an insoluble support and an enzyme(s)-labelled anti-chondrocalcin antibody, the insoluble support comprising mirror-finished beads having Ra less than 1.5 μm, wherein a protein of 16,000 to 50,000 molecular weight and 1.0 to 5.0 isoelectric point or a mixture containing said protein is allowed to exist so that the final concentration of said protein in the mixture is adjusted to 0.02-0.9% by weight in the immunoreaction solution.

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14. A monoclonal antibody which is characterised by that it recognises mammalian chondrocalcin.

PCT

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(54) Title: METHOD FOR ASSAYING CHONDROCALCINE

(54) 発明の名称 コンドロカルシンの測定方法

(57) Abstract

A method for assaying chondrocalcine by immunological technique using enzyme-labeled chondrocalcine or anti-chondrocalcine antibody of a mammal, which comprises using the body fluid of a mammal as a specimen and assaying chondrocalcine of the mammal utilizing immuno-reaction in a solution, is disclosed. Assaying reagents and kits for the method are also disclosed. The method, reagents and kits can be utilized for, e.g., diagnosis of cartilaginous diseases of mammals.

(57) 要約

酵素で標識した哺乳動物のコンドロカルシン又は抗コンドロカルシン抗体を用いる免疫学的測定方法において、検体として哺乳動物の体液を用い、かつ溶液中における免疫反応を利用して該哺乳動物のコンドロカルシンの量を測定することを特徴とするコンドロカルシンの測定方法、及びそれに用いる測定試薬，キット。

かかる測定方法等は、哺乳動物の軟骨性疾患の診断等に利用することができる。

情報としての用途のみ
PCTに基づいて公開される国際出願のパンフレット第1頁にPCT加盟国を同定するために使用されるコード

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TD チャード
TG トーゴ
US 米国

Field of the art:

The present invention relates to a method for determining chondrocalcin according to the immunoassay using an enzyme(s)-labeled mammalian chondrocalcin or enzyme(s)-labeled anti-chondrocalcin antibody, which is characterized by that a body fluid of mammalian is used as a specimen and the chondrocalcin of the mammalian is determined utilizing the immunoreactions in the fluid.

Moreover, the present invention relates to a reagent for determination and a kit therefor.

Chondrocalcin is a protein isolated from epiphyseal cartilage of fetal bovine in 1983 for the first time by H.U. Choi et al, and has been known to be a molecule of 69,000 molecular weight consisting of two subunits of 35,000 molecular weight [see H.U. Choi, et al., J. Biol. Chem. 258, 655 - 661, (1983)]. Subsequently, the chondrocalcin was identified to be C-propeptide of type II collagen by analysis of partial sequence of the amino acids [see M.V.D. Rest, et al., Biochem. J., 237, 923 - 925 (1986)]. The histochemical study has been made on the distribution of the protein in living body using bovines [see A.R. Poole et al., J. Cell Biol., 98, 54 - 65 (1984); Clinical Orthopaedics and Related Research, 208, 114 - 118 (1986)]. According to this study, the protein most distributes in cartilage where calcification is advancing, especially in the hypertrophic zone of the growth plate, while scarcely in mature bones, teeth and other tissues. The physiological action of this protein is presumed to participate closely in the calcification of

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cartilage, from the characteristic distribution in the living body and the fact that the protein binds to calcium ion and hydroxyapatite. In the course of these histochemical studies, immunological methods such as tissue staining have been applied and a polyclonal antibody obtained by immunizing a rabbit with bovine chondrocalcin has been employed to study bovine tissues.

In the meantime, there are few reports on the pathological study of chondrocalcin and future development can be expected. A.R. Poole et al. described that they are paying attention to chondrocalcin in connection with the pathology in the diseases relating to development, ossification of cartilage and abnormal calcification [see A.R. Poole et al., Clinical Orthopaedics and Related Research 208, 114 - 118 (1986)]. Therefore, the determination of chondrocalcin will play very important and significant roles in the basic medical and clinical studies on chondrocalcin.

A. Hinek et al. determined the chondrocalcin in the cartilage extracts and the supernatants of chondrocyte culture mixtures by the radioimmunoassay [A. Hinek et al., J. Cell. Boiol. 104, 1435 - 1441 (1987)]. The lowest limit of this measurement was, however, 100 ng per ml specimen and the sensitivity was not enough to determine chondrocalcin in body fluid such as blood or synovial fluid. Moreover, they described the measurement of only extracts of cartilages with 4M guanidine hydrochloride aqueous solution (at 5.8 pH) and supernatants of chondrocyte culture in the DME medium, but not the measurement of the body fluid containing the factors which would inhibit the specific reactions or induce non-specific reactions. Accordingly, the development of high-sensitivity measurement of chondrocalcin which can determine chondrocalcin in body fluid, and the reagents and kits for the measurement is very important to the diagnosis of diseases, monitoring of treatment from clinical point of

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view.

The immunoassay based on antigen-antibody reactions utilizes the specific reaction between an antigen and an antibody in body fluid containing many components. Actually, however, there happen non-specific reactions other than the target antigen-antibody reaction and inhibitory reactions, when body fluid is used as a specimen. Thus, when a known amount of an antigen to be measured is added to the specimen, the exact amount sometimes cannot be determined or the sensitivity necessary for measuring the antigen cannot be obtained. The causes of the reactions other than the specific one are not always be elucidated thoroughly, but it is understood that they happen, when compounds having antigenicity similar to that of the target substance are present, or in case of the presence of substances such as rheumatoid factor reacting with the Fc moiety of IgG, known as non-specific antibody. In laboratory examinations, therefore, the exact measurement of the target substance in body fluid is always an extremely important subject matter.

The present inventors have resolved the above-stated problems and achieved the invention which can determine chondrocalcin in body fluid.

Disclosure of the invention:

In other words, the present invention is a method for determining chondrocalcin according to immunoassay using enzyme(s)-labeled mammalian chondrocalcin or anti-chondrocalcin antibody, which is characterized by that mammalian body fluid is used as a specimen and the chondrocalcin of mammalian animals is determined utilizing immunoreactions in solutions.

Moreover, the present invention is a reagent for determining chondrocalcin in mammalian body fluid as a specimen, which is composed of an anti-chodrocalcin antibody reagent

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immobilized on an insoluble support and an anti-chondrocalcin antibody reagent labeled with an enzyme, and a kit for determining chondrocalcin in mammalian body fluid immunologically, which is composed of an anti-chondrocalcin antibody immobilized on an insoluble support, a labeled anti-chondrocalcin antibody, a solubilizer, a washing agent, a substrate for measuring the enzyme activity and a reaction terminator for the enzyme.

Moreover, the present invention is a monoclonal antibody which is characterized by that it recognizes mammalian chondrocalcin.

Brief explanation of the drawings:

Fig. 1 is a calibration curve for bovine chondrocalcin using a monoclonal antibody "Chon-5G" and another monoclonal antibody labeled with HRP "Chon-8G" as reagents for determination.

Fig. 2 is a calibration curve for bovine chondrocalcin using a monoclonal antibody "Chon-3F" and a polyclonal antibody labeled with HRP as reagents for determination.

Fig. 3 is a calibration curve for bovine chondrocalcin using a polyclonal antibody and a monoclonal antibody labeled with HRP "Chon-4H" as reagents for determination.

Fig. 4 is a calibration curve for determination of human chondrocalcin in a specimen.

Fig. 5 is a calibration curve for bovine chondrocalcin using reagents according to the present invention: anti-chondrocalcin polyclonal antibody and anti-chondrocalcin polyclonal antibody labeled with HRP.

Fig. 6 shows the effect of skim milk added to the chondrocalcin determination system. In Fig. 6, black dots and white dots give 5 ng/ml and 0 ng/ml of final chondrocalcin concentrations, respectively.

Fig. 7 is a calibration curve for bovine chondrocalcin

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using anti-chodrocalcin polyclonal antibody and anti-bovine-chondrocalcin polyclonal antibody, Fab', labeled with peroxidase as reagents.

Fig. 8 is a calibration curve for bovine chondrocalcin using anti-chondrocalcin polyclonal antibody and Fab' of anti-bovine-chondrocalcin monoclonal antibody, "Chon-4H", labeled with peroxidase.

Fig. 9 shows the effect of skim milk added to the chondrocalcin determination system using Fab'. In the Figure, the black dots and white dots give final bovine chondrocalcin concentration 1 ng/ml and 0 ng/ml, respectively.

Fig. 10 is a calibration curve for determination of human chondrocalcin in the specimens using anti-bovine-chondrocalcin polyclonal antibody and Fab' of anti-bovine-chondrocalcin polyclonal antibody labeled with peroxidase as reagents.

Fig. 11 is a calibration curve for determination of human chondrocalcin in body fluid using anti-bovine-chondrocalcin polyclonal antibody immobilized on mirror-finished polystyrene beads and $F(ab')_2$ of anti-bovine-chondrocalcin antibody labeled with peroxide.

Best embodiment of the Invention:

In the first place, the reagents for determining chondrocalcin according to the present invention will be described. The reagents according to the present invention comprise one reagent of anti-chondrocalcin antibody immobilized to an insoluble support and the other reagent of anti-chondrocalcin antibody labeled with an enzyme wherein such anti-chondrocalcin antibody is, for example, anti-chondrocalcin polyclonal antibody, or anti-chondrocalcin monoclonal antibody.

The chondrocalcin as an antigen for obtaining the polyclonal antibody used in the present invention is in

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principle of a natural type extracted from a natural substance, but may be of other types obtained, for example, through the protein engineering or biotechnology, as long as the chondrocalcins are immunologically equivalent to the natural one such as a hapten, namely the peptide containing the antigen determinants of the natural chondrocalcin.

As a material for obtaining chondrocalcin of the natural type, are cited, for example, cartilages of mammalian fetuses and those of mammalians at the growing stage, and the cartilages of bovine fetus are preferred, because they are available with relative easiness. The isolation and purification can be done by a combination of usual techniques for protein isolation such as salting-out, extraction, centrifugation, ultrafiltration, a variety of chromatography, and the method described by H.U. Choi is one of the examples.

The monoclonal antibody is prepared by culturing a hybridoma formed by the cell fusion technique according to G. Koeller and Milstein [Nature (London), 256, 495-497 (1975)] and isolating from the culture mixture containing the antibody excreted. In other words, a mouse is immunized with a chondrocalcin originating from a mammalian animal, and the mouse lymphocytes are fused with the mouse myeloma cells to give hybridomas. As the hybridomas given by this process produce a variety of monoclonal antibodies, the hybridoma producing the target monoclonal antibody is cloned whereby cloned hybridoma is isolated. The cloned hybridoma is cultured in vitro to excrete the monoclonal antibody. Anti-chondrocalcin antibody is separated from the supernatant of the culture mixture.

The polyclonal antibody is obtained through a usual process, for example, by a method described in the lecture course of experimental biochemistry, the second series, Vol. 5 page 1 to 10, edited by the Biochemical Society of Japan,

published by Tokyo Kagaku Dojin Co. Ltd. (Japan) (1986).

The animal to be immunized is not particularly limited, as long as it is a different mammal from the one from which chondrocalcin was obtained as an antigen. For example, when bovine chondrocalcin is used as an antigen, sheep, rabbits, Guinea pigs, rats or mice are preferably used.

When articular cartilages of human fetus and of normal adult were tissue-stained with the anti-bovine-chondrocalcin polyclonal antibody and the monoclonal antibody, the former gave an tinted image but the latter did not. This result evidently shows that the antibody according to the present invention recognizes human chondrocalcin.

In the present invention, the above-stated polyclonal antibody or monoclonal antibody is used at least in a labeled anti-chondrocalcin antibody. Additionally, when the anti-chondrocalcin antibody immobilized to an insoluble support or to be immobilized to an insoluble support is used, the combination can selected from the cases where both are polyclonal antibodies, and monoclonal antibodies and one is polyclonal and the other is monoclonal.

In some cases, Fab', $F(ab')_2$ or Facb fragments are preferred as the labeled antibody. In other words, first, there exists components bonding to the Fc moiety of the antibody such as the rheumatoid factor and such components cause reactions with non-specific antibodies other than the target antigen-antibody reaction. Consequently, exact determination of chondrocalcin in body fluid becomes difficult. When said fragment is used as the labeled antibody, however, such reactions do not happen and exact values are given. Secondly, in order to obtain sensitivity sufficient to measure satisfactorily chondrocalcin in specimens of body fluid, it is required that non-specific reactions to the insoluble support are reduced as much as possible, and said labeled fragment is preferred to use as the labeled anti-

body, because these non-specific reactions are inhibited.

Such fragment is obtained, in case of $F(ab')_2$ or Fab', by hydrolyzing the polyclonal or monoclonal antibody through a known procedure, for example, hydrolyzing with pepsin, or further subjecting F(ab')2 fragment to reductive treatment convert into Fab' fragment [see A. Nisonoff et al., Arch. Biochem. Biophys., 89, 230 (1960): P. Parham., J. Immunol., 131, 2895 (1983), and others].

Radioisotopes have been well known as a labeling substance, but their use is limited in the industrial production, because they may cause pollution, and have the time limit of use, further, the operators are required to undergo special training. Thus, enzymes are suitable for this purpose, because they have amplifying action without such defects as in radioisotopes.

As enzymes bonding to anti-chondrocalcin antibody, are cited, for example, lysozyme, maleate dehydrogenase, glucose-6-phosphate dehydrogenase, peroxidase, glucose oxidase, alkali phosphatase, luciferase, β-galactosidase, alcohol dehydrogenase, invertase. These enzymes are bonded to the anti-chondrocalcin antibody by a usual method such as the glutaraldehyde method, the periodate method or the maleimide method. For example, a maleimidized antibody or fragment of the antibody is allowed to react with a thiolated enzyme in a solvent. Maleimidization of an antibody or antibody fragment can be done using, for example, succinimidyl 4-(N-maleimidemethyl)cyclohexane carbonate (SMCC), sulfosuccinimidyl 4-(N-maleimidemethyl)cyclohexane carbonate (sulfoSMCC), succinimidyl-m-maleimidebenzoate (MBS), succinimidyl 6-maleimide hexanoate (EMCS). The introduction of mercapto groups into enzymes can be done by a known method, (for example, see enzyme immunoassay: edited by Ishikawa (Igakushoin) in Japanese). For example, it can be done by allowing an enzyme to react with S-acetylmercaptosuccinic

anhydride (AMSA) or N-succinimidyl-3-(2-pyridylthiopropionate (SPDP).

The labeling number of the enzyme in the bonded antibody obtained from an enzyme and anti-chondrocalcin antibody, namely in an enzyme(s)-labeled antibody can be determined by measurement of molecular weight, absorbance or enzyme activity. For example, when the enzyme is peroxidase, the absorbance at 280 nm wavelength originating from the antibody and peroxidase and the absorbance at 403 nm originating from peroxidase are measured to determine the labeling number (E. Ishikawa et al., J. Immunoassay., 4, 209 - 327 (1983), see page 243).

In other words, at 403 nm wavelength, there is no absorbance caused by the antibody and only by peroxidase, thus the concentration of peroxidase is calculated from the absorbance. Since the absorbance at 280 nm originates from both the antibody and peroxidase, the concentration of the antibody is calculated from the value subtracting the contribution of the peroxidase at 280 nm which is calculated from the concentration of peroxidase calculated from the absorbance at 403 nm and the molecular extinction coefficient of peroxidase at 280 nm from the absorbance at 280 nm, and from the molecular extinction coefficient of the antibody at 280 nm. Thus, the labeling number of peroxidase to the antibody can be obtained from the concentrations of peroxide and the antibody.

Thus, the labeling numbers of the labeled anti-chondrocalcin antibodies were determined and the correlation with measurement sensitivity was investigated. As a result, it was evidently shown that one molecule of Fab', $F(ab')_2$ or Facb fragment labeled with 1.0 or more molecules of enzyme is generally used, and the fragments labeled with 1.5 or more enzyme molecules (poly-labeled antibody) give more satisfactory results. In order to obtain sufficient measure-

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ment sensitivity to determine chondrocalcin in body liquid, the poly-labeled antibody is preferably composed of one molecule of Fab', $F(ab')_2$ or Facb fragment and more than 1.5 molecule of enzyme, more preferably more than 2 molecules of enzyme. The present invention, however, is not limited thereto.

Such poly-labeled antibody is produced by reaction of the above-mentioned maleimidized antibody or antibody fragment with a thiolated enzyme. As for the reaction conditions, the molar ratio of the fragment of said antibody to the said enzyme is 1 : 1 or more, preferably 1 : 3 or more; the reaction temperature is 4 to 40°C, preferably 4 to 30°C; the reaction pH, 5.5 to 7.5; and the reaction time, 5 - 48 hours. As long as the poly-labeled antibody according to the present invention is obtained, however, the reaction conditions are not limited to those cited above. The poly-labeled antibody obtained by such methods can be isolated and purified from the reaction m.xture by gel chromatography.

The enzyme-labeled antibody according to the present invention can exactly determine chondrocalcin in body fluid with sensitivity necessary for determination, as it inhibits nonspecific reactions and inhibitory reactions other than the objective antigen-antibody reaction, occurring when body fluid is used as a specimen.

On the other hand, anti-chondrocalcin antibody immobilized on an insoluble support is obtained as follows.

As an insoluble support, is cited naturally occurring polymers, their derivatives, synthetic polymers and their derivatives. As the former, are cited polysaccharides and their derivatives, for example, cellulose, Sephadex, Sepharose, carboxymethylcellulose, nitrocellulose, cellulose acetate, or dextran, or inorganic polymers such as glass or silica gel. As the latter, are mentioned vinyl polymers, such as polystyrene, polyethylene, polypropylene, ABS,

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polyvinyl fluoride, polyamine-methyl vinyl ether-maleic acid copolymer, ethylene-maleic acid copolymer, etc., polycondensation polymers such as polyamides, e.g., nylon 6, nylon 6/6, polyesters, e.g., polyethylene terephthalate, or amino acid polymers. The shape is not particularly limited to test tube, microtiter plate, beads, membrane, etc. The antibodies to be immobilized on the insoluble support are the molecule of anti-chondrocalcin antibody, its fragment maintaining the ability to bond to the antigen, for example, $F(ab')_2$, Fab', Fab, or Facb, or the derivatives of the antibody molecule holding the ability to bond to the antigen and its fragment. These antibodies are immobilized on the insoluble support by the physical adsorption, for example, dipping a support such as polystyrene in a solution of anti-chondrocalcin antibody; the ionic bonding, for example, using an ion-exchange resin or a support bearing ionizing functional groups, e.g., amino, carboxylic, sulfonic or phosphoric acid group; or the covalent bonding by chemical reactions, for example, the carboxyl chloride method, the carbodiimide method, the maleic anhydride derivative method, the cyanogen bromide-activated polysaccharide method, the diazo method, the activated ester method, the support-bonding method using a crosslinking agent (as a crosslinking agent, is used glutaraldehyde, hexamehtylene diisocyanate, succinimide, or maleimide, further the method for bonding through a substance which has no ability to bond to chondrocalcin, but can bond to anti-chondrocalcin antibody by biological reactions, for example, a method using a protein A-bonded support.

The use of an insoluble support whose surface is mirror-finished is preferred, because nonspecific adsorption is reduced, while measurement sensitivity is increased. As such a mirror-finished insoluble support, is cited a body having the surface of less than 1.5 μm centerline average roughness

(Ra). The centerline average roughness can be measured by means of a surface roughness-measuring apparatus, for example, Surfcom 570A (Tokyo Precision Co. Ltd.)

Such a mirror-finished insoluble support is not especially limited, as long as the Ra is less than 1.5 m, but, for example, polystyrene or the like is preferably used.

When the anti-chondrocalcin antibody immobilized on the insoluble support according to the present invention which has been prepared as stated above is used, a trace amount of chondrocalcin can be determined exactly in body fluid as a specimen.

Subsequently, the method for measuring chondrocalcin according to the present invention will be illustrated, which is characterized by using the reagent prepared by the procedures stated above and mammalian body fluid as a specimen to determine the chondrocalcin in the mammalian by utilizing the immune reactions in the solution.

There are so-called the competition method and the sandwich method in the immunological measurement methods according to the present invention. The sandwich method includes the one-step process and the 2-step process. In the one-step sandwich process, the antigen-antibody reaction between the specimen including the antigen to be determined, the solid-phase antibody immobilized on an insoluble support and an enzyme-labeled antibody is carried out in the same reaction system to form the solid-phase antibody-antigen-labeled antibody complex, followed by washing and determination of the labeled substance. In this case, the reaction may be conducted either in the coexistence of the specimen, the solid-phase antibody and the labeled antibody or sequentially the reaction between the specimen and the solid-phase antibody may be followed by addition of the labeled antibody for reaction. Or the reaction of the specimen with the labeled antibody may be followed by addition of the solid-

phase antibody. Any procedures can be adopted, as long as the solid-phase antibody-antigen-labeled antibody complex is formed before washing. On the other hand, in the two-step sandwich method, the reaction of the specimen with the solid-phase antibody is first conducted to form the solid-phase antibody-antigen complex, then the remaining specimen is removed and washed off, and the labeled substance is added to form the solid-phase antibody-antigen-labeled antibody complex. After washing, the labeled substance is determined.

In the present invention, the two-step process can be suitably applied to the cases where the specimen of body fluid contains the substances which disturb the measurement or the those which cause nonspecific reactions. In this process, after reaction of the antigen in the specimen with the solid-phase antibody, the specimen is removed and washed, and the labeled antibody is allowed to act, thus the reaction of the labeled antibody is not influenced by the above-stated substances with the accuracy and sensitivity increased. In the meantime, the two-step process has problems of more complicated operations in comparison with the one-step process, for example, the increased times of washing operations and the prolonged operation time. The one-step sandwich process also can be adopted. This process is one of preferred embodiments, because it is simple in operations and short in the reaction time, but it is generally susceptible to body fluid. In other words, the sensitivity of the process is lowered or becomes insufficient by the nonspecific reactions and inhibition of the immune reactions.

In the present invention, for example, when a mirror-finished, insoluble support, especially beads having the surface of less than 1.5 μm centerline average roughness, are used as an insoluble support, and a multi-labeld $F(ab')_2$

is employed as a labeled antibody, nonspecific adsorption to said beads is inhibited and the sensitivity is increased by the multi-labeled antibody $F(ab')_2$, whereby the one-step sandwich process becomes adequately possible.

In the determination method according to the present invention, it is preferred that a protein of 16,000 to 50,000 molecular weight and 1.0 to 5.0 isoelectric point or a mixture thereof is added to the immune reaction solution, until the final concentration reaches 0.02 to 0.9 % by weight in the immune reaction solution, because the unspecific adsorption is inhibited, and consequently the back ground is remarkably lowered and the sensitivity is increased. Such protein or a mixture thereof can be added to the immunoassay reagent according to the present invention until the concentration of the protein reaches a prescribed value. The protein is, for example, casein, pepsin, oviglycoprotein, orosomucoid or the like. Such mixture can contain 10 to 60 % by weight, preferably 20 to 50 % by weight of the above-stated protein, 30 to 80 % by weight, preferably 40 - 60 % by weight of saccharide, e.g., lactose, other lipids (for example, 0.5 - 2 % by weight), ash (for example, 5 - 12 % by weight) and water (for example, 2 to 8 % by weight). One of the typical mixtures is skim milk. Skim milk contains casein as a protein and characteristically shows a better dispersion ability in the immune reaction solution, higher NBS (Non-specific binding ) effect per gram of protein and better storability (resistant to form precipitation) at 4°C, than in case of casein only. In the present invention, any defatted milk can be used as a skim milk according to the present invention, regardless of its origination. One of the most typical one is the commercially available skim milk produced by Difco Company.

In the present invention, the molecular weight of protein means the value measured according to the osmotic

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pressure method, in more detail, the molecular weight is measured by utilizing the fact that the difference in osmotic pressure can be a parameter for the molecular weight of the protein, said pressure difference between the high polymer solution and the pure solvent both of which is separated with a semipermeable membrane which can pass through the solvent, but not the high polymer. In present invention, 6.66 M urea solution is used and the osmotic pressure was measured at 4°C. The isoelectric point means the value measured according to the chromatographic focussing method where the protein is separated at the isoelectric point, in more detail, a column (measuring 0.5 cm diameter x 45 cm length) is filled with PBE94 gel (Pharmacia Co. Ltd.) and 0.025 M imidazole hydrochloride buffer solution (pH 7.4) is used for elution.

Any kinds of usual solvents can be used as a solvent for the immune reaction, as long as they do not cause adverse effect on the reaction. For example, phosphate buffer solution, tris hydrochloride buffer solution, acetate buffer solution at 6.0 to 8.0 pH are preferred.

The temperature of the immune reaction at the measurement is not especially limited, as long as the constituent protein is not denatured, and the immune reaction is not markedly inhibited, however, in general, the reaction is satisfactorily conducted lower than 50°C, preferably at about 4°C to about 45°C for about 5 minutes to about 20 hours.

The mammalian body fluid for the measurement according to the present invention means the body fluid of mammalian such as human or equine containing factors inhibiting the specific reactions and causing non-specific reactions and may be in the form of usual clinical samples, for example, blood in the form of serum or plasma, joint fluid, lymph, thymic fluid, ascitic fluid, amniotic fluid, cell tissue

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fluid, myeloid fluid or urine.

In the present invention, the anti-chondrocalcin antibody immobilized on the insoluble support and labeled anti-chondrocalcin antibody can be combined with other substances necessary for the immunoassay to give a kit for determining chondrocalcin. The other substances for immunoassay are, for example, a solubilizer, a washing agent, a substrate for measuring the enzyme activity and a reaction terminator, which are known.

Thus, according to the present invention, a sample containing a trace amount of mammalian chondrocalcin such as a clinical sample, is used as a specimen, to determine the chondrocalcin in the sample in high sensitivity and accuracy through simple operations.

The possibility of industrial application:

The values of chondrocalcin in body fluid which are determined using the immunoassay and the reagent according to the present invention are useful in diagnosis and monitoring the treatment of cartilage diseases of mammalian animals, for example, horse arthropathy or human chondrocyte-relating diseases. Especially, it can be used for diagnosis and treatment monitoring of human osteoarthritis and discriminative diagnosis between osteoarthritis and rheumatoid arthritis.

The present invention will be illustrated in detail by the examples and comparisons. In the examples, % means percent by weight.

Example 1

(1) Preparation of bovine chondrocalcin

According to the method by H. U. Choi, 10.0 mg of bovine chondrocalcin were obtained from the cartilage of 20

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long tubular bones of bovine fetal limbs. The SDS-PAGE electrophoresis gave a single band of 35,000 molecular weight.

(2) Preparation of antigen-stimulating lymphocytes

An emulsion of 90 µg of bovine chondrocalcin and Freund's complete adjuvant was given intraperitoneally to male B alb/c mouse. After that, an emulsion of 40 to 50 µg of bovine chondrocalcin and Freund's incomplete adjuvant were given 5 times at 3 to 4-week intervals. On the tenth day after the 5th administration, 60 µg of chondrocalcin were dissolved in 1.0 ml of physiological saline solution and given intravenously. The spleen was enucleated aseptically 4 days later, passed through a mesh made of stainless steel to give a spleen cell suspension in the RPMI-1640 medium supplemented with 0.39 g/l of L-glutamine, 0.2 g/l of Kanamycin sulfate and 2.0 g/l of sodium hydrogen carbonate. Said suspending cells were washed 3 times with the medium to give the spleen cell suspension.

(3) Cell fusion

Mouse myeloma cells P3U1 were cultured in the GIT synthetic medium (DAIGO EIYO Chemical Co. Ltd.) supplemented with 0.2 g/l of Kanamycin sulfate. The myeloma cells were on the logarithmic phase. The spleen cells and the myeloma cells were suspended in a serum-free RPMI-1640 medium at a cell number ratio of 3 : 1 and centrifuged at 1,300 rpm for 5 minutes. After removal of the medium, the cell precipitate was suspended, as 50 % solution of polyethylene glycol of 1,500 average molecular weight (7.2 pH) was quietly added by 1 ml. The suspension was combined with 9 ml of the serum-free RPMI-1640 medium, and the cells were carefully stirred. The mixture was centrifuged at 1,000 rpm for 5 minutes, the supernatant was removed, the cell precipitate was collected and suspended in 40 ml of HAT medium.

Cloned hybridoma

The fused cell suspension was distributed on the 96 microplate (200 µl every well). The plate was cultured at 37°C in 5 % CO atmosphere. The half amount of the medium was exchanged with the virgin HAT medium 1 day and two days later, and the operation was repeated at 2-day interval. After 11 days, the antibody against bovine chondrocalcin in the supernatant of hybridoma culture mixture was screened by the enzyme immunoassay. The antigen used in the screening was bovine chondrocalcin and the second antibody was alkali-phosphatase-labeled goat anti-mouse IgG antibody.

Among 192 wells to which the hybridoma was distributed, the colonies of the fused cells were observed in 102 wells and the 7 wells in the 102 wells were antibody-positive. The hybridomas in the antibody-positive wells were diluted so that every well of the 96 microplate includes 0.9 cell. Then, Balb/c mouse thymus cells were distributed to the microplates as feeder cells and they were cultured in the GIT culture medium supplemented with 0.2 g/l of kanamycin sulfate. The single colony was confirmed by microscopic observation. The antibody against bovine chondrocalcin in the supernatant of the hydridoma culture was screened by the enzyme immunoassay. Each well was found to be antibody-positive and produce anti-bovine chondrocalcin monoclonal antibody.

Culture of hybridoma

These cloned hybridomas were injected into the abdominal cavities $10^6$ - $10^7$ cells/every Balb/c mouse to which 0.1 ml of pristane (WAKO Pure Chemical Co. Ltd.) had been given intraperitoneally 2 weeks ago. Then, ascites was collected 2 to 3 ml/mouse on the 7th to 10th day.

Purification of monoclonal antibody

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The collected ascites was purified according to the method by Ey et al [see P.L. Ey et al., Immunochemistry, 15, 429 -436 (1978)]. In other words, 2 to 3 ml of the ascites fluid was fed to a protein A-Sepharose column (gel volume: 5 ml) equilibrated with 0.1 M phosphate buffer solution (pH 8.0) and the monoclonal antibody was eluted using, in turn, 0.1 M sodium citrate buffer solutions of 6.0, 5.0, 4.0 and 3.0 pH to collect the purified monoclonal antibody, namely Chon-3F, Chon-5G, Chon-8G and Chon-4H.

(7) Class of purified monoclonal antibodies

The specific class of the purified monoclonal antibodies was determined by the Ouchterlony gel diffusion test using the class-specific anti-mouse antiserum. The results are given in Table 1.

Table 1

| Antidody | $IgG_1$ | $IgG_2a$ | $TgG_2b$ | $IgG_3$ | IgM |
|---|---|---|---|---|---|
| Chon.-3F | - | + | - | - | - |
| Chon.-5G | - | - | + | - | - |
| Chon.-8G | - | + | - | - | - |
| Chon.-4H | - | + | - | - | - |

(8) Search on whether the recognition sites by the monoclonal antibodies are indentical or diffrent

The bovine chondrocalcin PBS solution of 2 µg/ml was distributed to 96 microplate 150 µg/l every well, allowed to stand one overnight at 4°C, washed with PBS, combined with 0.5% BSA-PBS, and left to stand further one overnight, whereby an antigen-immobilized plate was prepared. This plate was combined with 150 µl of 5 µg/ml monoclonal anti-

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body PBS solution (the first antibody) in each well and they were allowed to stand for 75 minutes at 37°C. After the wells were washed, 0.5 % solution of the monoclonal antibody labeled with horse raddish peroxidase (the sencond antibody) in BSA-PBS was added to the wells 150 µl every well and they were allowed to react at 37°C for 60 minutes. After rinsing with physiological saline solution, the HRP substrate solution (containing 50 mg/dl of 2,2'-azino-di[3-ethylbenzthiazolinesulfonate (6)]diammoniuim salt and 50 µl/dl of 2M hydrogen peroixde in 0.1 M phosphate/citrate buffer solution (pH 4.5) was added by 150 µl to emit fluorescence at 25°C for 6 minutes. The reactions were terminated with 50 µl of 0.1 M aqueous oxalic acid and the measurement was carried out using a plate reader at 415 nm wavelength.

It is obvious from the results shown in Table 2, that the four monoclonal antibodies are three kinds of antibodies having different epitopes, and Chon-5G and Chon-4H have the same or close epitope and cannot bind simultaneously.

Table 2

| First antibody | Second antibody | Absorbance |
|---|---|---|
| Chon.-3F | | 0.450 |
| Chon.-5G | Chon.-4H | 0.198 |
| Chon.-8G | | 0.503 |
| Chon.-4H | | 0.185 |
| Chon.-3F | | 0.195 |
| Chon.-5G | Chon.-5G | 0.002 |
| Chon.-8G | | 0.189 |
| Chon.-4H | | 0.001 |

Example 2

An emulsion of 1 mg of bovine chondrocalcin purified in Example 1 and Freund's complete adjuvant was subcutaneously given to rabbits. After 19 days, 450 µg of bovine chondrocalcin was subcutaneously administered in the same manner. After additional 16 days, an emulsion of 450 µg of bovine chondrocalcin and Freund's complete adjuvant was subcutaneously given. On the 10th day after the final administration, the blood was collected, prepared into serum and subjected to enzyme immunoassay. In other words, bovine chondrocalcin was used as an antigen, and horse radish peroxidase-labeled goat anti-rabbit IgG antibody, as the second antibody, to measure the antibody value against bovine chondrocalcin and it was found to 400,000 folds. Then, the whole blood was collected to prepare serum and the serum was precipitated with 40% saturated ammonium sulfate solution, and purified with protein A-Sepharose-4B column to obtain the polyclonal antibody against bovine chondrocalcin.

Example 3

Chon.-5G, the monoclonal antibody described in Example 1 was dissolved in 0.1 M phosphate/citrate buffer solution (pH 4.0) in 20 µg/ml concentration, and the solution was distributed to a microplate 100 µl every well and immobilized by allowing it to stand at 4°C for 24 hours. The product was washed with PBS, combined with 0.5 % BSA-PBS 100 µl every well, allowed to stand one overnight at 4°C to effect after-coating, finally washed with PBS whereby an Chon.-5G immobilized plate was obtained.

Subsequently, a dilution series of the purified bovine chondrocalcin, 15, 10, 5, 1.5 and 0 ng/ml was prepared using PBS, added to the Chon.-5G-immobilized plate 100 µl every

well, and allowed to react at 37°C for 1 hour. After washing with PBS, 100 µl of HRP substrate (TMB) was added to develop the color at 37°C for 30 minutes, then the reaction was stopped by adding 100 µl of 1N sulfuric acid solution, and the absorbance was measured with a plate reader at 450 nm wavelength.

The resulting calibration curve is given in Fig. 1.

Example 4

Chon.-3F, the monoclonal antibody described in Example 1, was dissolved in 0.1 M phosphate/citrate buffer solution so that the protein concentration became 20 µg/ml, then polystyrene beads were dipped in the solution 3 days and nights whereby the antibody was immobilized to the beads. After being washed with PBS, the beads were dipped in BSA-PBS one overnight to effect after-coating, rinsed with PBS again to give Chon.-3F-immobilized beads.

A dilution series of the purified bovine chondrocalcin, 15, 10, 5, 1.67 and 0 ng/ml, was prepared using PBS and these solutions were allowed to react with the Chon.-3F-immobilized beads at 37°C for 1 hour. After being rinsed with PBS, 0.5 % solution of horse radish peroxidase (HRP)-labeled polyclonal antibody (described in Example 2) in BSA-PBS was added to the beads in an amount of 400 µl and they were allowed to react at 37°C for 1 hour. After being washed with PBS, 400 µl of HRP substrate (containing 0.125 % of 3,3',5,5'-tetramethylbenzidine and 0.03 5 of hydrogen peroxide in 0.1 M phosphate/citrate buffer solution (pH 4.5) was added to emit the light at 37°C for 30 minutes. The reaction was terminated with 1.0 ml of 1N-sulfuric acid and the absorbance was measured with a spectrophotometer at 450 nm wavelength .

The resultant calibration curve is given in Fig. 2.

Example 5

According to the process described in Example 4, beads to which the polyclonal antibody against bovine chondrocalcin (described in Example 2) was immobilized were prepared.

Then, a dilution series of purified bovine chondrocalcin 15, 10, 5, 1.67 and 0 ng/ml was prepared using PBS and 400 µl each was added to a glass tube and allowed to react with the immobilized beads at 37°C for 1 hour. After rinsing with PBS, 400 µl of 0.5 % Chon.-4H (described in Example 1), HRP-labeled monoclonal antibody solution in BSA-PBS was allowed to react at 37°C for 1 hour. As in Example 4, the calibration curve of Fig. 3 was obtained.

Example 6

A dilution series of purified bovine chondrocalcin, 10.0, 5.0, 1.0, 0.6, 0.4, 0.2 0.1 and 0.0 ng/ml was prepared using PBS. Each bovine chondrocalcin was combined with normal human plasma which had been passed through a column filled with Sepharose-4B to which the polyclonal antibody against bovine chondrocalcin (prescribed in Example 2) was immobilized, in an equal volume to prepare a series of dilution system containing chondrocalcin 5.0, 2.5, 0.5, 0.3, 0.2, 0.1, 0.05, 0.0 ng/ml in two-fold diluted human plasma, respectively.

The dilution series of bovine chondrocalcin solutions was measured using Chon.-4H (described in Example 1)-immobilized plate which had been prepared as in Example 3 and the HRP-labeled polyclonal antibody (prescribed in Example 2) to make a calibration curve of Fig. 4.

Subsequently, serum or synovial fluid was combined with an equal volume of PBS, respectively and the absorbance of the 2-fold diluted samples was measured as in Example 3, and the concentration of human chondrocalcin converted into bovine chondrocalcin was calculated from the calibration

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curve in Fig. 4. The results are summarized in Table 3.

Table 3

| No. | Diseases | Fluid | Absorbance | Concentration |
|---|---|---|---|---|
| 1 | Normal | serum | 0.028 | 0.0 (ng/ml) |
| 2 | osteosarcoma | serum | 0.062 | 1.1 |
| 3 | osteoarthritis | synovial fluid | 0.093 | 2.0 |
| 4 | osteoarthritis | synovial fluid | 0.333 | 9.0 |

Example 7

The anti-bovine chondrocalcin polyclonal antibody described in Example 2 was dissolved in 0.1 M phosphate/citrate buffer solution (pH 4.0) to form 20 µg/ml solution and the solution was distributed on a microplate 100 µl every well and immobilized by leaving it to stand at 4°C for 24 hours. The products were washed with PBS, after-coated by adding 0.5% BSA-PBS 100 µl every well and allowing them to stand one overnight at 4°C, and rinsed with PBS, to give bovine chondrocalcin polyclonal antibody-immobilized plates.

Then, a dilution series of purified bovine chondrocalcin, 15, 10, 5, 1.5, 0 ng/ml was prepared using 1 % BSA-final concentration 0.065% skim milk-PBS solution and distributed to the antibody-immobilized plate 100 µl every well so that they were allowed to react at 37°C for 1 hour. They were washed with PBS, 0.5% solution of HRP-labeled anti-bovine chondrocalcin polyclonal antibody in 0.5 % BSA-0.1% skim milk-PBS solution was distributed 100 µl every well to effect the reaction at 37°C for 1 hour. After PBS washing, the substrate for HRP (containing 2.5 mM hydrogen peroxide

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and 0.0225 % 3,3', 5,5'-tetramethylbenzidine) was added to individual wells 100 µl every well to develop the color at 37°C for 30 minutes. Then, 100 µl of 1N-sulfuric acid was added to terminate the reaction, then the absorbance was measured with a plate reader at 450 nm wavelength.

The resultant calibration curve is shown in Fig. 5.

The lower limit of the measurement was 1 ng/ml and it was markedly higher sensitivity than that of the radioimmunoassay according to A. Hinek et al., 100 ng/ml [see J. Cell Biol., 104, 1435-1447 (1987)].

Example 8

Bovine chondrocalcin was diluted with the buffer solution for the immunoassay (1% BSA-containing 0.01 M phosphate and 0.85% NaCl buffer solution (pH = 7.2), abbreviated to 1% BSA-PBS hereinafter) to prepare 0 and 10 ng/ml solutions. The solution, 50 µl, was thoroughly mixed with 50 µl of skim milk solutions of 0, 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 % final concentrations in the same buffer solution, then the solution was distributed to the microplate to which the anti-bovine chondrocalcin polyclonal antibody described in Example 2 had been immobilized 100 µl every well to conduct the reaction at 37°C for 2 hours (the first reaction). Then, the reaction mixture was washed with PBS-0.05% Tween-20, the HRP-labeled anti-bovine chondrocalcin antibody which had been prepared as described in Example 3, is diluted with a buffer solution for immunoassay containing skim milk whose final concentration becomes the same as in the first reaction, and the resultant solution was added to each well by 100 µl to conduct the reaction at 37°C for 1 hour (the second reaction). After rinsing with PBS-0.05 % Tween-20, the substrate for HRP described in Example 3 was added by 100 µl to develop the color at 37°C for 30 minutes, then 1N sulfuric acid was added by 25 µl to terminate the

reaction. The absorbance was measured with a plate reader at 450 nm wavelength. The results are given in Fig. 6. As shown evidently from the figure, skim milk can inhibits the non-specific adsorption at a concentration higher than 0.00625 %.

Example 9

The Fab' fragment of anti-bovine chondrocalcin polyclonal antibody (Anti-chondrocalcin PCA) described in Example 2 was allowed to bond to peroxidase according the process based on the Nisonoff's method to prepare a peroxide-labeled Fab' (see the lecture course of experimental biochemistry, the second series, Vol. 5 page 109 to 112, edited by the Biochemical Society of Japan, published by Tokyo Kagaku Dojin Co. Ltd. (Japan) (1986). In other words, 1250 µg of anti-chondrocalcin PCA was dissolved in 1.25 ml of 0.2 M sodium acetate buffer solution (pH 4.5) and dialyzed against the buffer solution. Pepsin 25 µg was added to digest the dialyzate at 37°C for 24 hours. Sodium hydroxide 1N solution was added dropwise to the reaction mixture until the pH rose up to 8 to terminate the reaction. The $F(ab')_2$ fragment of 92,000 molecular weight was separated from the reaction mixture through TSK gel 3000SW column-HPLC using 5 mM EDTA-0.1M sodium phosphate buffer solution (pH 6.0) as an eluent. The product was concentrated by ultrafiltration to collect 2.2 ml of $F(ab')_2$ solution in 122.3 µg/ml concentration. The product was combined with 245 µl of 0.1M 2-mercaptoethylamine to effect reductive treatment at 37°C. The reaction mixture was concentrated by ultrafiltration and the Fab' fragment of 46,000 molecular weight was separated through HPLC as in $F(ab')_2$. 2.0 ml of Fab' fragment solution of 83.7 µg/ml concentration was obtained.

In the meantime, 4.0 mg of horse radish peroxidase (Toyo Spinning Co. Ltd.; abbreviated to HRP) was dissolved

in 0.6 ml of 0.1M sodium phosphate buffer solution (pH 7.0), 40 µl of N-succinimidyl-3-maleimide benzoate solution in dimethylformamide (31.4 mg/ml concentration ) was added dropwise thereto to conduct the reaction at 30°C for 1 hour. The reaction mixture was fed into a Sephacryl S-200 column and eluted with 0.1 M sodium phosphate buffer solution (pH 6.0) to separate maleimide-HRP.

Then, 470 µl of 3.10 mg/ml maleimide-HRP was added dropwise to the above-stated Fab' fragment solution to conduct the reaction at 4°C for 20 hours. The reaction mixture was concentrated by ultrafiltration, and HRP-labeled Fab' of 86,000 molecular weight was obtained by the TSK gel 3,000 SW column-HPLC eluted with PBS (pH 7.2). The labeled Fab' was used to make the following calibration curves.

The anti-chondrocalcin PCA described in Example 2 was dissolved in 0.1 M phosphate/citrate buffer solution (pH 3.5) to prepare a solution of 20 µg/ml concentration. The solution was distributed to a microplate 100 µl every well and immobilized by allowing to stand at 4°C for 24 hours. After being rinsed with PBS, 1.0 % BSA-PBS was added 100 µl every well and left to stand at 4°C for 24 hours to form the after coat, then rinsed with PBS to give anti-chondrocalcin PCA-immobilized plates.

A dilution series of purified bovine chondrocalcin, 0, 10, 50, 100, 500, 1,000, 5,000, 10,000 pg/ml was prepared using 1 % BSA-final concentration 0.1 % skim milk-PBS (pH 7.2) and they were added to the antibody-immobilized plate 100 µl every well to carry out the reaction at 37°C for 5 hours, and additionally at 4°C for 24 hours. The reaction mixture was washed with PBS-0.05 % Tween-20, then diluted with 1.0 % BSA-final concentration 0.1 % skim milk-PBS (pH 7.2) so that the concentration of the Fab' component in the HRP-labeled anti-chondrocalcin PCA Fab' became 60 ng/ml. The prepared labeled Fab' solution was distributed 100 µl every

well to conduct the reaction at 20°C for 4 hours. The reaction mixture was rinsed with PBS-0.05% Tween-20, then a substrate for HRP (containing 2.5 mM of hydrogen peroxide and 0.0225 % of 3,3',5,5'-tetramethylbenzidine) to develop the color at 20°C for 2 hours. Then, 25 µl of 1N-sulfuric acid solution was added to terminate the reaction and the absorbance was measured with a plate reader at 450 nm wavelength.

The calibration curve in Fig. 7 was obtained from the results.

As evidently shown in Fig. 7, the lower limit of the measurement was 10 pg/ml and considerably higher sensitive than that of the radioimmunoassay, 100 ng/ml according to Hinek et al [see J. Cell. Biol. 104, 1435-1441 (1981)].

Example 10

The Fab' fragment of Chon.-4H, anti-bovine chondrocalcin MCA, described in Example 1, was prepared according to the Parham's method [see the lecture course of experimental biochemistry, the second series, Vol. 5 page 100, edited by the Biochemical Society of Japan, published by Tokyo Kagaku Dojin Co. Ltd. (Japan) (1986)] and HRP-labeled Fab' was obtained in the same way as in Example 8. In other words, 480 µg of Chon.-4H was dissolved in 0.1 M sodium citrate buffer solution (pH 4.1) to prepare a solution of 2.0 ml. Then, the solution was dialyzed against the same buffer solution. The product was digested with 56.25 µg of pepsin at 37°C for 90 minutes. To the reaction mixture, 1N NaOH was added dropwise until the pH rose up to 8 to terminate the reaction. The reaction mixture was subjected to TSK gel 3,000 SW column-HPLC using 5 mM EDTA-0.1M sodium phosphate buffer solution (pH 6.0) to separate $F(ab')_2$ fragment of 92,000 molecular weight. The product was concentrated to give 7.5 ml of $F(ab')_2$ solution of 19.6 µg/ml concentration.

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Then, 835 µl of 0.1 M 2-mercaptoethanolamine was added to effect reductive treatment at 37°C for 2 hours. From this reaction mixture, 5.5 ml of Fab' fragment solution of 9.5 µg/ml concentration was obtained as in Example 9.

Subsequently, 146 µl of 3.10 mg/ml of maleimide-HRP was added dropwise to the Fab' fragment solution to conduct the reaction at 4°C for 20 hours. HRP-labeled Fab' was obtained form the reaction mixture in the same way as in Example 8. The labeled Fab' was used to provide the following calibration curve.

The immobilized plate used here was prepared in Example 9.

A dilution series of purified bovine chondrocalcin, 0, 10, 50, 100, 500, 1,000 5,000 and 10,000 pg/ml was prepared using 1 % BSA 0.1% skim milk-PBS (pH 7.2) and distributed to the antibody-immobilized plate 100 µl every well to conduct the reaction at 37°C for 2 hours. After being washed with PBS-0.05 % Tween 20, the HRP-labeled Fab' obtained from Chon.-4H was diluted with 1 % BSA-final concentration 0.1 % skim milk-PBS (pH 7.2) so that the concentration of the Fab' component became 300 ng/ml, and the prepared labeled Fab' solution was distributed 100 µl every well to effect the reaction at 37°C for 1 hour. After rinsing with PBS-0.05 % Tween 20, the substrate for HRP (containing 2.5 mM hydrogen peroxide and 0.0255 % of 3,3',5,5'-tetramethylbenzidine) was added 100 µl every well to develop the color at 20°C for 30 minutes, then 25 µl of 1N sulfuric acid was added to terminate the reaction, and the absorbance was measured with the plate reader at 450 nm wavelength.

Fig. 8 shows the calibration curve obtained from the result. The lower limit of the measurement was 50 pg/ml, and the sensitivity was considerably higher than that in the method according to A. Hinek, et al., 100 ng/ml.

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Example 11

Bovine chondrocalcin was diluted with the buffer solution for immunoassay (1 % BSA-containing 0.01 M phosphate 0.85% NaCl buffer solution (pH 7.2), abbreviated to 1 % BSA-PBS hereinafter) to prepare 0.2 ng/ml solution. Then, 50 ml of the solution was thoroughly mixed with 50 µg of solutions containing skim milk in the final concentrations of 0, 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 %. Then, 100 ml of the solution was distributed to the anti-chondrocalcin PCA-immobilized plate prepared as in Example 8 to effect the reaction at 37°C for 2 hours (the first reaction). After rinsing with PBS-0.05 % Tween-20, the HRP-labeled Fab' prepared as in Example 8 was diluted with a buffer solution for immunoassay containing skim milk so that the final concentration became equal to that in the first reaction, then distributed 100 ml every well to effect the reaction at 37°C for 1 hour (the second reaction). After rinsing with PBS-0.05 % Tween-20, the substrate for HRP described in Example 8 was added by 100 µl to develop the color at 37°C for 0.5 hour, and 25 µl of 1N sulfuric acid was added to terminate the reaction. The absorbance was measured with a plate reader at 450 nm wavelength. The results are given in Fig. 9. As shown evidently in the figure., non-specific adsorption can be inhibited with skim milk in the concentration higher than 0.00625 %.

Example 12

A dilution series of purified bovine chondrocalcin, 0, 7.5, 37.5, 75, 375, 750, 3,750, and 7,500 pg/ml was prepared using 1% BSA-final concentration 0.00625 % skim milk-PBS (pH 7.2). The each solution was combined with 1/4 volume of normal human plasma which had been passed through a column filled with anti-chondrocalcin PCA-immobilized Sepharose 4B to prepare a dilution series of bovine chondrocalcin con-

taining 5-fold diluted human plasma. In the dilution series, bovine chondrocalcin is included in plasma, 0, 30, 150, 300, 1,500, 3,000, 15,000, and 30,000 pg/ml.

The bovine chondrocalcin in the dilution series was determined using the polyclonal antibody-immobilized plate and the HRP-labeled Fab' which had been prepared in the same way as in Example 9 to draw the calibration curve in Fig. 10.

Then, a specimen of serum or synovial fluid was combined with 4-fold volume of 1 % BSA-final concentration 0.00625 % skim milk-PBS (pH 7.2) to prepare the 5-fold diluted specimen. Then, the absorbance was measured as in Example 9 and human chondrocalcin converted from bovine chondrocalcin was calculated from the calibration curve in Fig. 10.

The results are summarized in Table 4.

As shown clearly in the calibration curve in Fig. 10, the lower limit of the measurement according to the present invention is 30 pg/ml and showed markedly higher sensitivity than that of the radioimmunoassay according to A. Hinek et al., of 100 ng/ml lower limit.

Table 4

| No. | Diseases | Fluid | Absorbance | Concentration |
|---|---|---|---|---|
| 1 | Osteoarthritis | Serum | 0.054 | 450 pg/ml |
| 2 | Osteosarcoma | Serum | 0.086 | 1,300 |
| 3 | Osteoarthritis | Synovial F. | 0.110 | 2,050 |
| 4. | Osteoarthritis | Synovial F. | 0.229 | 6,200 |

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Example 13

Preparation of peroxidase-labeled anti-chondrocalcin $F(ab')_2$-labeled antibody

1. Preparation of the $F(ab')_2$ fragment of anti-chondrocalcin rabbit polyclonal antibody

Pepsin 0.1 mg was added to 2.5 ml of a solution of anti-chondrocalcin rabbit polyclonal antibody described in Example 2 (6.0 mg) in an acetate buffer solution (pH 4.5), they were incubated at 37°C one overnight, and subjected to HPLC whose pH had been adjusted to 6.0 with 1N NaOH (the column: TSK gel G-3,000SW, the eluent: PBS pH 7.2) to collect 3.6 mg of $F(ab')_2$.

2. Maleimidization of $F(ab')_2$

$F(ab')_2$ fragment (3.6 mg) was dissolved in PBS, and 0.09 ml of a solution of succinimidyl-m-maleimide benzoate (MBS) solution in DMF (10 mg/ml) was added dropwise to the $F(ab')_2$ solution at 25°C. After agitation for 30 minutes, the solution was subjected to column chromatography using a column of 1 cm x 45 cm filled with Sephadex G-25 and 0.1 M phosphate buffer solution as an eluent to collect 4.0 mg of maleimidized $F(ab')_2$ fragment.

3. Introduction of the thiol group of peroxidase

An S-acetylmercaptosuccinic anhydride solution in DMF (6.0 mg/ml), 0.2 ml, was gradually added dropwise to 5.4 ml of a peroxidase (Toyo Spinning Co. Ltd.: 54 mg) solution in 0.1 M phosphate buffer (pH 6.5) and the reaction was continued for additional 1 hour. The solution was combined with 2.1 ml of 0.1 M tris hydrochloric acid buffer (pH 7.0), 0.4 ml of 0.1 M EDTA solution and 4.3 ml of 1M hydroxylamine solution, then they were stirred at 30°C for 5 minutes. The resultant solution was placed in a dialysis tube, and dia-

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lyzed against 0.1M phosphate buffer solution (pH 6.0)-5 mM EDTA at 4°C for two overnights to give a peroxidase solution including SH groups (2.54 mg/ml).

4. bonding of the maleimidized $F(ab')_2$ fragment to thiol-containing peroxidase.

The maleimidized $F(ab')_2$, 4.0 mg, and the thiol-containing peroxidase solution (2.54 mg/ml), 2.8 ml, mixed and left to stand one overnight at 25°C. The reaction mixture was subjected to HPLC (column: TSK Gel G-3,000SW ) using PBS (pH 7.2) as an eluent to collect the fractions of peroxidase-bonded $F(ab')_2$ whereby $F(ab')_2$ was obtained by 3.1 mg.

The molar ratio of the $F(ab')_2$ fragment to the peroxidase was 1 : 2.7 in the fraction.

Example 14

Anti-chondrocalcin polyclonal antibody described in Example 2 was dissolved in 0.01 M phosphate-buffered physiological saline solution (abbreviated to PBS hereinafter) so that the concentration was adjusted to 20 µg/ml and mirror-finished polystyrene beads [Immunochemical : immunobeads having 1.3 µm surface centerline average roughness (Ra) measured with Surfcom 570A (Tokyo Precision Co. Ltd.)] were dipped in the solution and allowed to stand at 4°C for one overnight. The beads were rinsed with PBS 3 times, then allowed to stand in 1 % BSA-PBS (pH 7.2) at room temperature for 2 hours. The beads were rinsed again with PBS 3 times, and stored in PBS at 4°C until they were used.

Solutions of chondrocalcin in 0.75 % BSA-0.01 % skim milk-PBS (pH 7.2) were prepared, varying the concentrations as follows: 0, 0.025, 0.3125, 0.625, 1.25, 5 ng, and these solutions were placed in small plastic tubes made of polypropylene 0.2 ml each. Then, the peroxide-labeled anti-chondrocalcin $F(ab')_2$ fragment, which was prepared in Exam-

ple 13, was dissolved in 0.5 % BSA-0.2 % skim milk-PBS (pH 7.2) so that the concentration became 3 μg/ml as the $F(ab')_2$, and the solution was added to the individual tubes 0.2 ml each. Immediately after addition, the anti-chondrocalcin immobilized beads were added to the tubes one bead each and they were incubated at 37°C for 90 minutes.

The beads were washed with a solution of PBS containing 0.05 % Tween 20 (abbreviated to PBS-T) 3 times, then 2.3 mM hydrogen peroxide-0.025 % 3,3',5,5'-tetramethylbenzidine solution was added to the tubes 0.4 ml each to incubate at 37°C for 30 minutes. Then, 1 ml of 1N sulfuric acid was added to terminate the color reaction, and the absorbance was determined at 450 nm wavelength.

The resultant calibration curve are shown in Fig. 11. The lower limit was 25 pg/ml, and the sensitivity was markedly higher than that in the radioimmunoassay according to A. Hinek et al., 100 ng/ml [see A. Hinek et al., J. Cell. Biol. 104, 1435-1441 (1987)].

Subsequently, specimens of serum or synovial fluid were diluted with 3-fold volume of 0.75 % BSA-0.01 % skim milk-10 mM PBS (pH 7.2) to prepare 4-fold diluted specimens and their absorbance was measured as in drawing the above calibration curve. Thus, human chondrocalcin converted from bovine chondrocalcin was determined from the calibration curve in Fig. 11.

The results are given in Table 5.

Table 5

| No. | Diseases | Fluid | Absorbance | Concentration |
|---|---|---|---|---|
| 1 | Osteoarthritis | Synovial F. | 0.580 | 5.0 ng/ml |
| 2 | Osteoarthritis | Synovial F. | 0.775 | 7.1 " |
| 3 | Osteoarthritis | Synovial F. | 0.310 | 2.4 " |
| 4 | Osteoarthritis | Synovial F. | 0.395 | 3.4 " |
| 5 | Osteoarthritis | Synovial F. | 0.325 | 2.5 " |
| 6 | Osteoarthritis | Synovial F. | 1.005 | 9.9 " |
| 7 | Osteoarthritis | serum | 0.153 | 0.9 " |
| 8 | Chronic R. A.* | Synovial F. | 0.085 | 0.2 " |
| 9 | Chronic R. A. | Synovial F. | 0.090 | 0.3 " |
| 10 | Chronic R. A. | Synovial F. | 0.105 | 0.5 " |

Note: * Chronic R. A. = Chronic Rheumatoid Arthritis

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS.

1. A method for determining chondrocalcin by a sandwich immunoassay using an anti-chondrocalcin antibody immobilised to an insoluble support and an enzyme(s)-labelled anti-chondrocalcin antibody, the insoluble support comprising mirror-finished beads having Ra less than 1.5 μm, wherein a body fluid of mammalian is used as a specimen and the chondrocalcin of mammalian is determined utilising an immunoreaction in solution and a protein of 16,000 to 50,000 molecular weight and 1.0 to 5.0 isoelectric point or a mixture containing said protein is allowed to exist so that the final concentration of said protein in the mixture is adjusted to 0.02-0.9% by weight in the immunoreaction in solution.

2. The method for determining chondrocalcin according to claim 1, wherein the immunoreaction is carried out in one system containing the anti-chondrocalcin antibody immobilised on the insoluble support, the enzyme(s) labelled anti-chondrocalcin antibody and the body fluid of mammalian as the specimen.

3. The method for determining chondrocalcin according to claim 1, wherein at least one of the anti-chondrocalcin antibody immobilised on an insoluble support and enzyme(s) labelled anti-chondrocalcin antibody is a monoclonal antibody recognising human chondrocalcin which is obtained using a mammalian chondrocalcin as an immunogen.

4. The method for determining chondrocalcin according to claim 1, wherein at least one of the anti-chondrocalcin antibody immobilised on an insoluble support and enzyme(s) labelled anti-chondrocalcin antibody is a polyclonal antibody recognising human chondrocalcin which is obtained by immunising the mammalian with chondrocalcin from a different kind of mammalian.

5. The method for determining chondrocalcin according to claim 1, wherein

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the enzyme(s) labelled anti-chondrocalcin antibody is the Fab' fragment or $F(ab')_2$ fragment of anti-chondrocalcin antibody.

6. The method for determining chondrocalcin according to claim 1, wherein the enzyme(s) labelled anti-chondrocalcin antibody is an anti-chondrocalcin antibody labelled with 1.5 or more molecules of enzyme per one molecule of the antibody.

7. The method for determining chondrocalcin according to claim 1, wherein the mixture containing said protein is skim milk.

8. A chondrocalcin-determining reagent for measuring immunologically the chondrocalcin in a body fluid of mammalian as a specimen, comprising an anti-chondrocalcin antibody immobilised on an insoluble support and an enzyme(s)-labelled anti-chondrocalcin antibody, the insoluble support comprising mirror-finished beads having Ra less than 1.5 μm, wherein a protein of 16,000 to 50,000 molecular weight and 1.0 to 5.0 isoelectric point or a mixture containing said protein is allowed to exist so that the final concentration of said protein in the mixture is adjusted to 0.02-0.9% by weight in the immunoreaction solution.

9. The reagent for determining chondrocalcin according to claim 8, wherein at least one of the anti-chondrocalcin antibody immobilised on an insoluble support and the enzyme(s) labelled anti-chondrocalcin antibody is a monoclonal antibody recognising human chondrocalcin which is obtained using a mammalian chondrocalcin as an immunogen.

10. The reagent for determining chondrocalcin according to claim 8, wherein at least one of the anti-chondrocalcin antibody immobilised on an insoluble support and the enzyme(s) labelled anti-chondrocalcin antibody is a polyclonal antibody recognising human chondrocalcin which is obtained by immunising the mammalian with chondrocalcin from a different kind of mammalian.

11. The reagent for determining chondrocalcin according to claim 8, wherein the enzyme(s) labelled anti-chondrocalcin antibody is the Fab' fragment or $F(ab')_2$ fragment of anti-chondrocalcin antibody.

12. The reagent for determining chondrocalcin according to claim 8, wherein the enzyme(s) labelled anti-chondrocalcin antibody is an anti-chondrocalcin antibody labelled with 1.5 or more molecules of enzyme per one molecule of the antibody.

13. The reagent for determining chondrocalcin according to claim 8, wherein the mixture containing said protein is skim milk.

14. A monoclonal antibody which is characterised by that it recognises mammalian chondrocalcin.

15. The monoclonal antibody according to claim 14, wherein it is obtained by immunising a mouse using bovine chondrocalcin as an immunogen and said mammalian is human.

16. The monoclonal antibody according to claim 14, wherein it is obtained by immunising a mouse with a peptide hapten containing the antigenic determinant of natural chondrocalcin of mammalian as an immunogen.

DATED this 7th day of September, 1992

Teijin Limited
by its Patent Attorneys
DAVIES COLLISON CAVE

FIG. 1

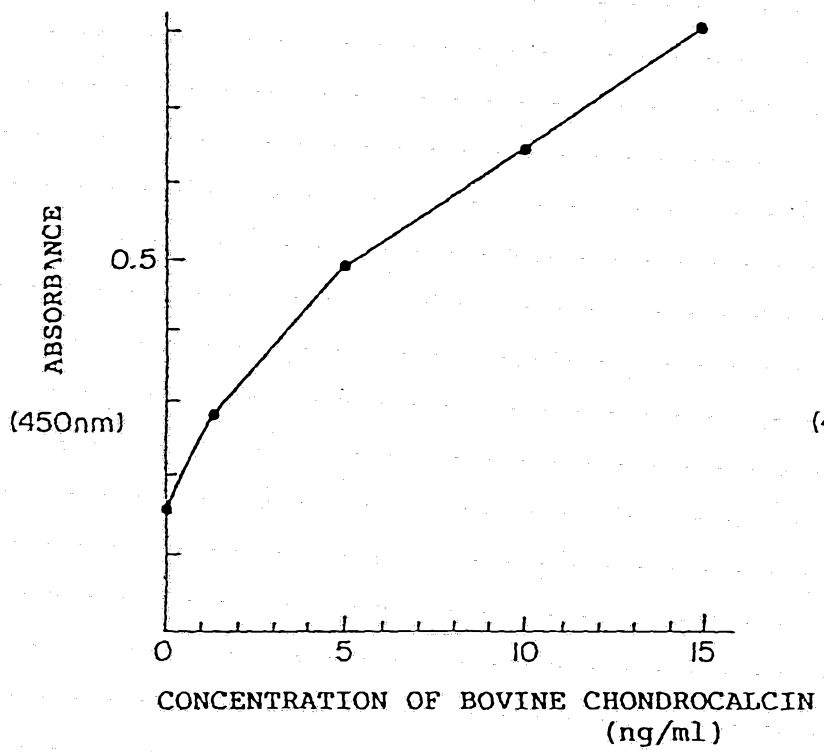

ABSORBANCE
(450nm)
0.5
0
5
10
15
CONCENTRATION OF BOVINE CHONDROCALCIN
(ng/ml)

FIG. 2

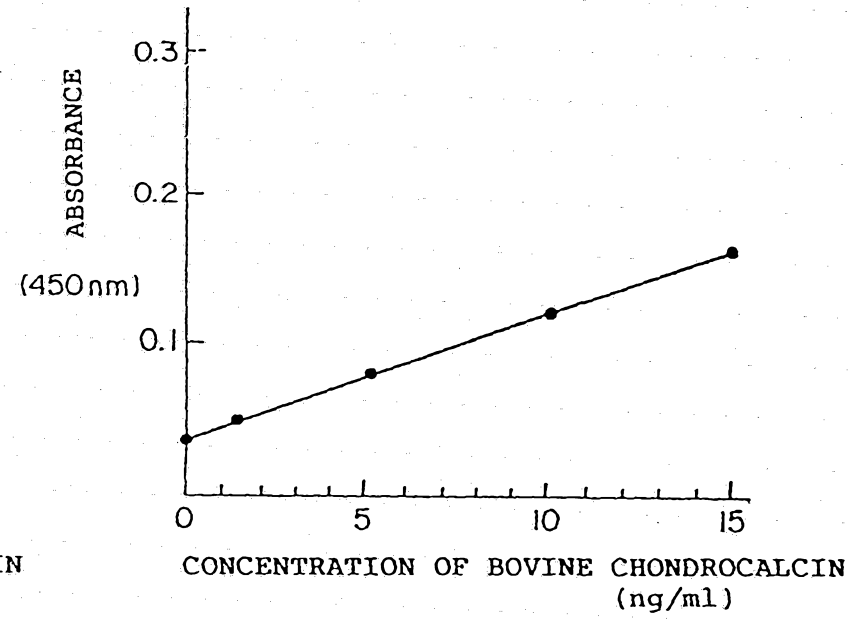

ABSORBANCE
(450nm)
0.3
0.2
0.1
0
5
10
15
CONCENTRATION OF BOVINE CHONDROCALCIN
(ng/ml)

FIG. 3

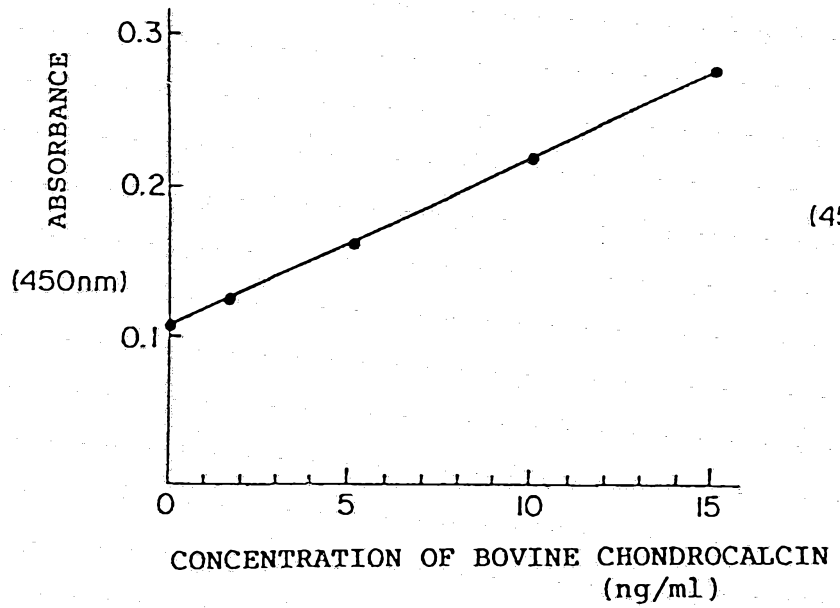

ABSORBANCE
(450nm)
0.3
0.2
0.1
0
5
10
15
CONCENTRATION OF BOVINE CHONDROCALCIN
(ng/ml)

FIG. 4

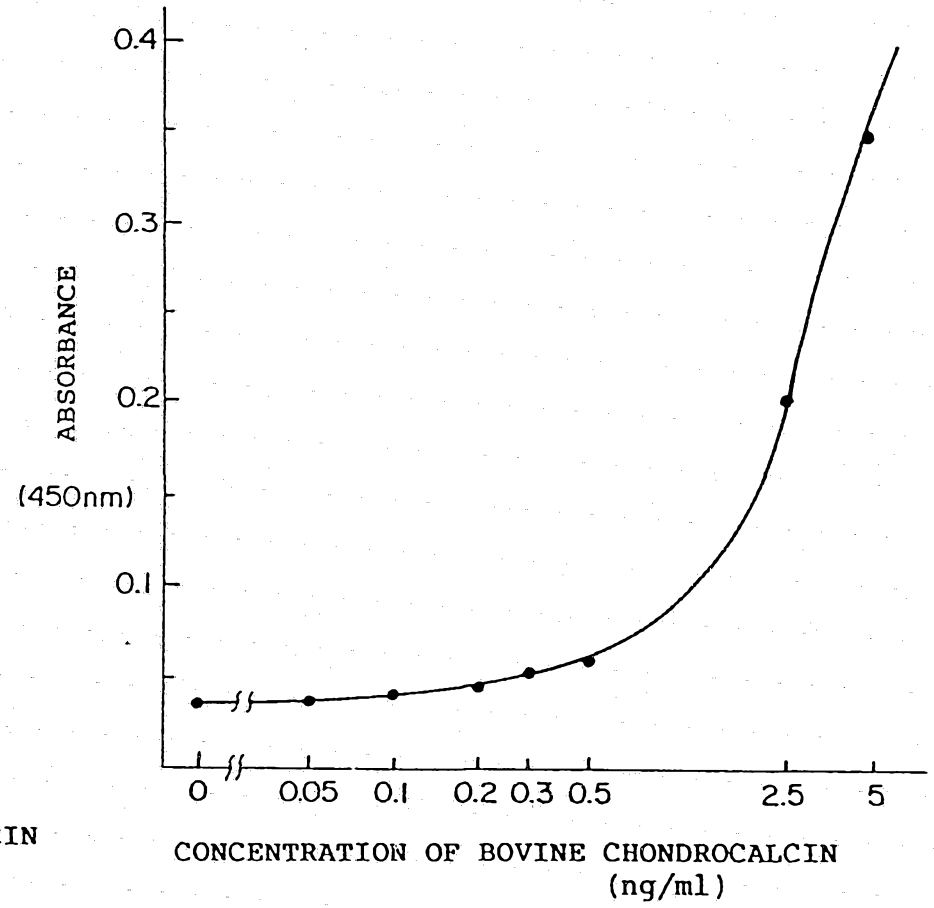

ABSORBANCE
(450nm)
0.4
0.3
0.2
0.1
0
0.05
0.1
0.2
0.3
0.5
2.5
5
CONCENTRATION OF BOVINE CHONDROCALCIN
(ng/ml)

FIG. 5

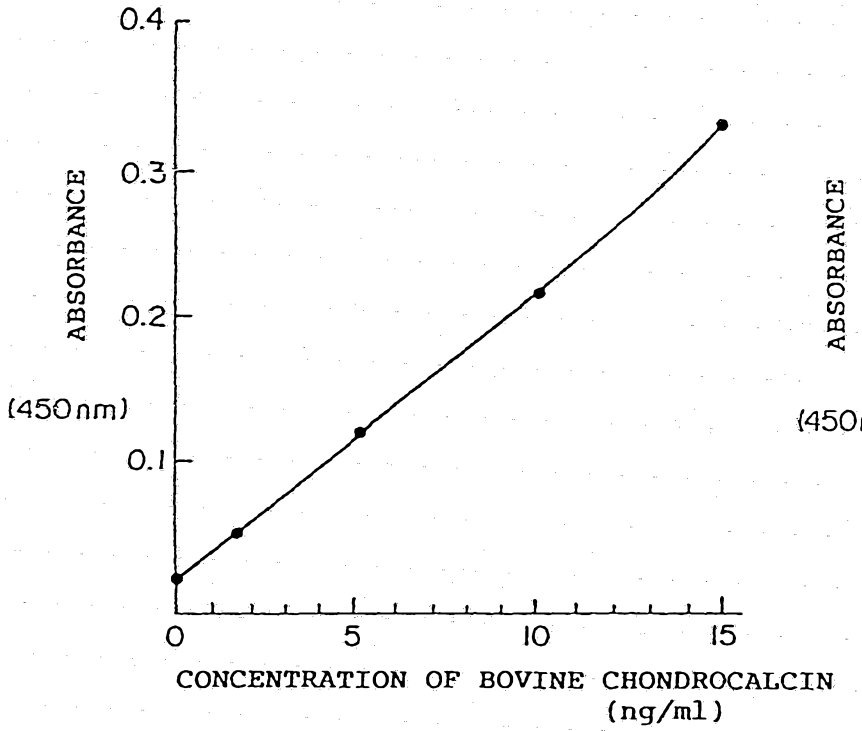

0.4
0.3
0.2
0.1
ABSORBANCE
(450nm)
0
5
10
15
CONCENTRATION OF BOVINE CHONDROCALCIN
(ng/ml)

FIG. 6

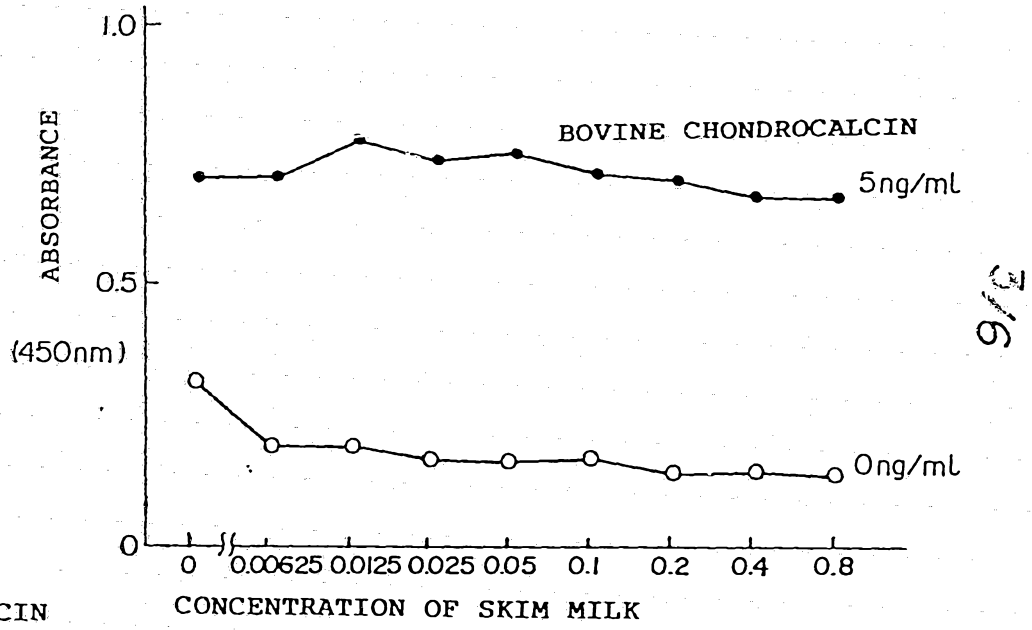

1.0
0.5
0
ABSORBANCE
(450nm)
BOVINE CHONDROCALCIN
5ng/ml
0ng/ml
0
0.00625
0.0125
0.025
0.05
0.1
0.2
0.4
0.8
CONCENTRATION OF SKIM MILK

FIG. 7

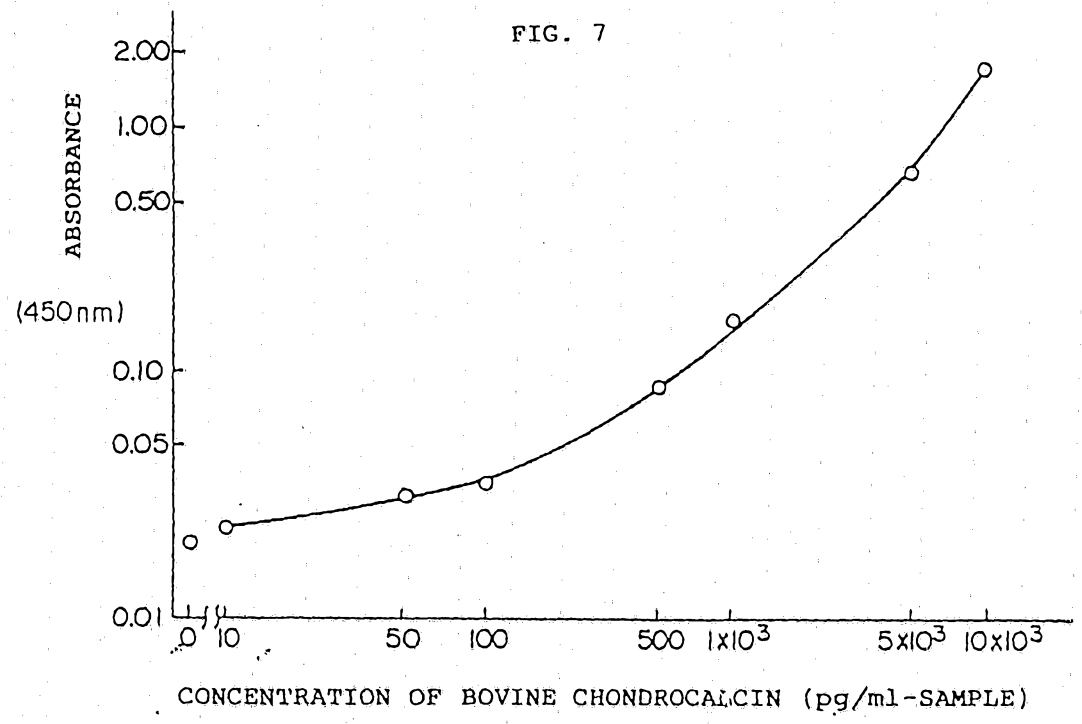

ABSORBANCE
(450nm)
2.00
1.00
0.50
0.10
0.05
0.01
0
10
50
100
500
1x10³
5x10³
10x10³
CONCENTRATION OF BOVINE CHONDROCALCIN (pg/ml-SAMPLE)

FIG. 8

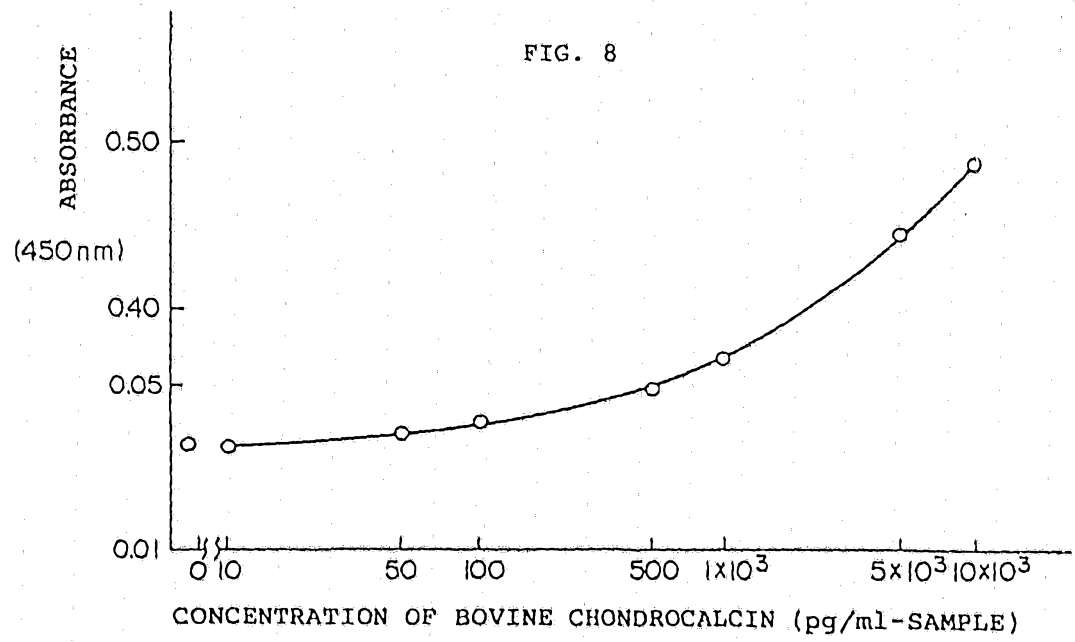

ABSORBANCE
(450nm)
0.50
0.40
0.05
0.01
0
10
50
100
500
1x10³
5x10³
10x10³
CONCENTRATION OF BOVINE CHONDROCALCIN (pg/ml-SAMPLE)

FIG. 9

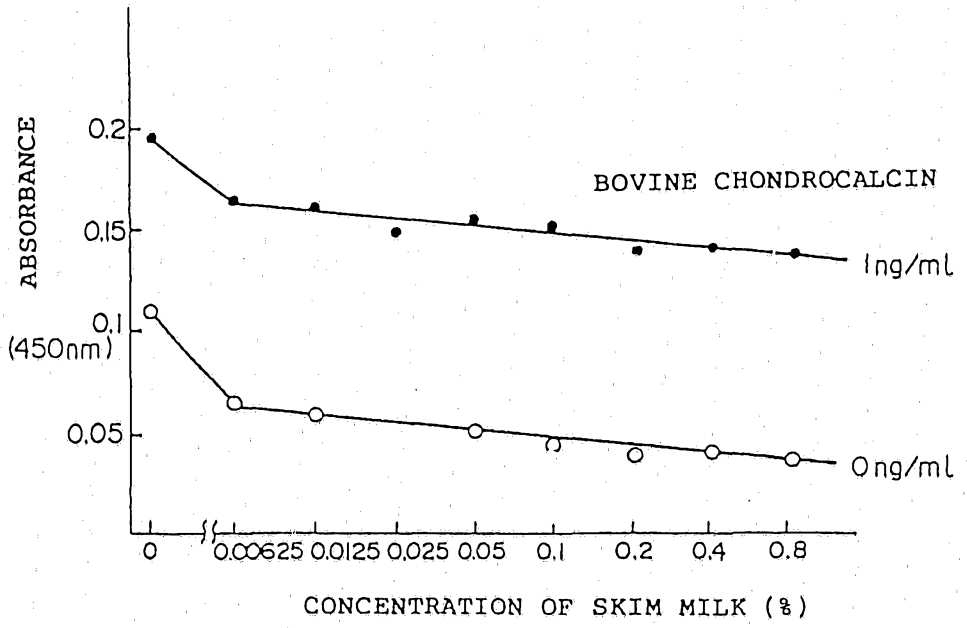

ABSORBANCE
(450nm)
0.2
0.15
0.1
0.05
BOVINE CHONDROCALCIN
1ng/ml
0ng/ml
0
0.00625
0.0125
0.025
0.05
0.1
0.2
0.4
0.8
CONCENTRATION OF SKIM MILK (%)

FIG. 10

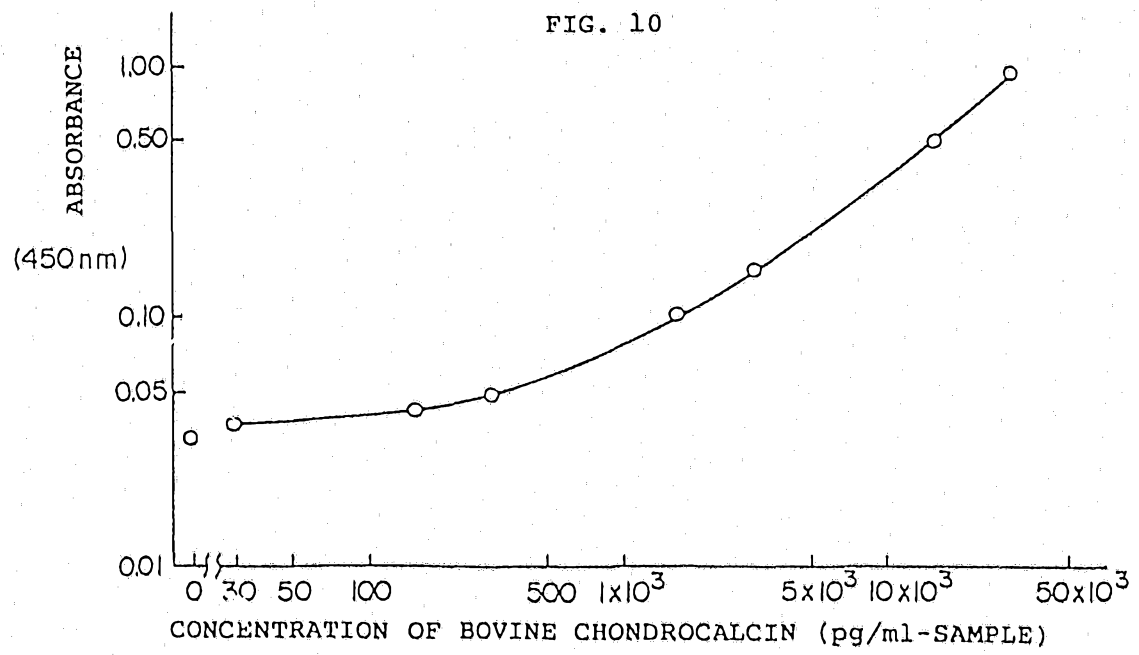
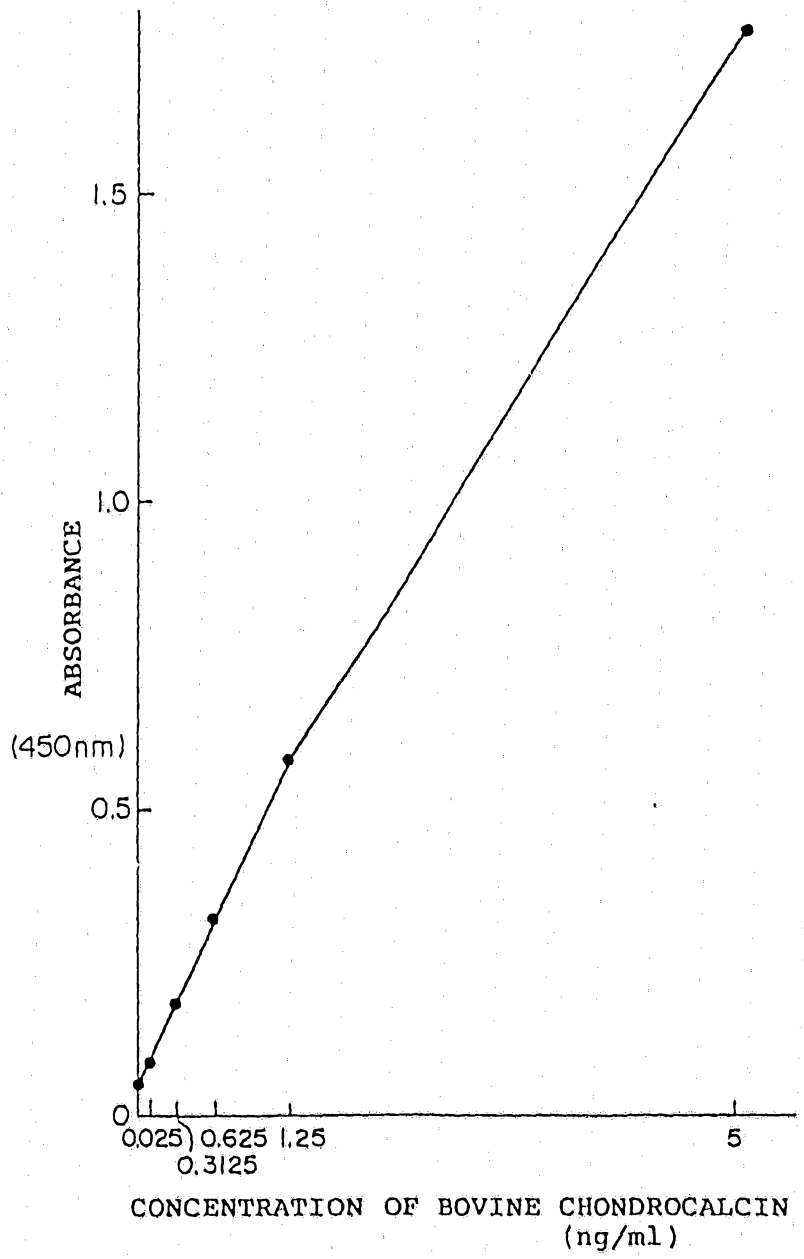

ABSORBANCE
(450nm)
1.00
0.50
0.10
0.05
0.01
0
30
50
100
500
1x10³
5x10³
10x10³
50x10³
CONCENTRATION OF BOVINE CHONDROCALCIN (pg/ml-SAMPLE)

FIG. 11


1.5
1.0
ABSORBANCE
(450nm)
0.5
0
0.025
0.3125
0.625
1.25
5
CONCENTRATION OF BOVINE CHONDROCALCIN
(ng/ml)

# INTERNATIONAL SEARCH REPORT

International Application No PCT/JP89/00138

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl[4] G01N33/53, G01N33/543, G01N33/577

**II. FIELDS SEARCHED**

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| IPC | G01N33/53, G01N33/543, G01N33/577 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

| | |
|---|---|
| Jitsuyo Shinan Koho | 1971 - 1988 |
| Kokai Jitsuyo Shinan Koho | 1971 - 1988 |

**III. DOCUMENTS CONSIDERED TO BE RELEVANT** [9]

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| X<br>Y | The Journal of Cell Biology, Vol.98, No.1, (1984), A.R. Poole et al. [Association of an Extracellular Protein (Chondrocalcin) with the Calufication of Cartilage in Endochondral Bone Formation] P.54-65 | 1-19<br>20-22 |
| X<br>Y | The Journal of Cell Biology, Vol.104, No.5, (1987), Aleksander Hinek et al. [The Calcification of Cartilage Matrix Chondrocyte Culture : Studies of the C-Propeptide of Type II Collagen (Chondrocalcin)] P.1435-1441 | 1-19<br>20-22 |
| A | J. Anat., Vol. 150, (1987), E. Liune et. al. [Age-related changes in the role of matrix vesicles in the mandibular condylar cartilage] P.61-74 | 1-22 |

* Special categories of cited documents: [10]

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

"E" earlier document but published on or after the international filing date

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

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

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

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

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

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

"&" document member of the same patent family

**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| April 27, 1989 (27. 04. 89) | May 15, 1989 (15. 05. 89) |
| **International Searching Authority** | **Signature of Authorized Officer** |
| Japanese Patent Office | |

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

| | | |
|---|---|---|
| Y | JP, A, 62-116262 (Takara Shuzo Co., Ltd.)<br>27 May 1987 (27. 05. 87)<br>(Family: none) | 20-22 |
| Y | JP, A, 62-24149 (Collagen Corporation)<br>2 February 1987 (02. 02. 87)<br>(Family: none) | 20-22 |
| Y | JP, A, 60-130825 (Fujisawa Pharmaceutical Co., Ltd.)<br>28 June 1985 (28. 06. 85)<br>& EP, A, 138222 & GB, A, 8327860 | 20-22 |

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

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

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

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

3. ☐ Claim numbers ....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

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

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

# 国 際 調 査 報 告

国際出願番号PCT/JP 89/ 00138

I．発明の属する分野の分類

国際特許分類（IPC） Int. Cl⁴
G01N33／53, G01N33／543, G01N33／577

II．国際調査を行った分野

調査を行った最小限資料

| 分類体系 | 分類記号 |
|---|---|
| IPC | G01N33／53, G01N33／543, G01N33／577 |

最小限資料以外の資料で調査を行ったもの

日本国実用新案公報　1971－1988年
日本国公開実用新案公報　1971－1988年

III．関連する技術に関する文献

| 引用文献のカテゴリー※ | 引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示 | 請求の範囲の番号 |
|---|---|---|
| X<br>Y | The Journal of Cell Biology, 第98巻, 第1号, (1984), A.R.Poole et al. 「Association of an Extracellular Protein (Chondrocalcin) with the Calufication of Cartilage in Endochondral Bone Formation」 P.54－65 | 1－19<br>20－22 |
| X<br>Y | The Journal of Cell Biology, 第104巻, 第5号, (1987), Aleksander Hinek et al. 「The Calcification of Cartilage Matrix Chondrocyte Culture : Studies of the C－Propeptide of Type II Collagen (Chondrocalcin)」 P.1435－1441 | 1－19<br>20－22 |
| A | J.Anat., 第150巻, (1987), E. Liune | 1－22 |

※引用文献のカテゴリー
「A」特に関連のある文献ではなく、一般的技術水準を示すもの
「E」先行文献ではあるが、国際出願日以後に公表されたもの
「L」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献（理由を付す）
「O」口頭による開示、使用、展示等に言及する文献
「P」国際出願日前で、かつ優先権の主張の基礎となる出願の日の後に公表された文献
「T」国際出願日又は優先日の後に公表された文献であって出願と矛盾するものではなく、発明の原理又は理論の理解のために引用するもの
「X」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの
「Y」特に関連のある文献であって、当該文献と他の1以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの
「&」同一パテントファミリーの文献

IV．認　　証

| 国際調査を完了した日 | 国際調査報告の発送日 |
|---|---|
| 27. 04. 89 | 15.05.89 |
| 国際調査機関 | 権限のある職員 |
| 日本国特許庁（ISA/JP） | 特許庁審査官　秋　月　美紀子　2G 7906 |

様式PCT／ISA／210（第2ページ）（1981年10月）

第2ページから続く情報

| | | |
|---|---|---|
| | （Ⅲ欄の続き）<br><br>et al.「Age-related changes in the role of matrix vesicles in the mandibular condylar cartilage」P.61-74 | |
| Y | JP, A, 62-116262（宝酒造株式会社）27.5月.1987（27.05.87）（ファミリーなし） | 20-22 |
| Y | JP, A, 62-24149（コラーゲンコーポレーション）2.2月.1987（02.02.87）（ファミリーなし） | 20-22 |

V. ☐ 一部の請求の範囲について国際調査を行わないときの意見

次の請求の範囲については特許協力条約に基づく国際出願等に関する法律第8条第3項の規定によりこの国際調査報告を作成しない。その理由は、次のとおりである。

1. ☐ 請求の範囲＿＿＿＿は、国際調査をすることを要しない事項を内容とするものである。

2. ☐ 請求の範囲＿＿＿＿は、有効な国際調査をすることができる程度にまで所定の要件を満たしていない国際出願の部分に係るものである。

3. ☐ 請求の範囲＿＿＿＿は、従属請求の範囲でありかつPCT規則6.4(a)第2文の規定に従って起草されていない。

VI. ☐ 発明の単一性の要件を満たしていないときの意見

次に述べるようにこの国際出願には二以上の発明が含まれている。

1. ☐ 追加して納付すべき手数料が指定した期間内に納付されたので、この国際調査報告は、国際出願のすべての調査可能な請求の範囲について作成した。
2. ☐ 追加して納付すべき手数料が指定した期間内に一部分しか納付されなかったので、この国際調査報告は、手数料の納付があった発明に係る次の請求の範囲について作成した。
請求の範囲＿＿＿＿
3. ☐ 追加して納付すべき手数料が指定した期間内に納付されなかったので、この国際調査報告は、請求の範囲に最初に記載された発明に係る次の請求の範囲について作成した。
請求の範囲＿＿＿＿
4. ☐ 追加して納付すべき手数料を要求するまでもなく、すべての調査可能な請求の範囲について調査することができたので、追加して納付すべき手数料の納付を命じなかった。

追加手数料異議の申立てに関する注意

☐ 追加して納付すべき手数料の納付と同時に、追加手数料異議の申立てがされた。
☐ 追加して納付すべき手数料の納付に際し、追加手数料異議の申立てがされなかった。

Ⅲ. 関連する技術に関する文献（第2ページからの続き）

| 引用文献のカテゴリー* | 引用文献名及び一部の箇所が関連するときは、その関連する箇所の表示 | 請求の範囲の番号 |
|---|---|---|
| Y | JP, A, 60－120825（藤沢薬品工業株式会社）<br>28. 6月. 1985（28. 06. 85）<br>&EP, A, 138222 &GB, A, 8327860 | 20－22 |