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SPECIFICATION

HARD TISSUE INTACTLY DISSOLVED MATERIALS AND METHOD FOR PRODUCING THE SAME

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Field of the invention

The present invention relates to hard tissue
intactly dissolved materials and method for producing
the materials, and more particularly, to functional
food materials ingestible to the human body, or novel
10 materials applicable to feed, medical products,
cosmetics, etc., and a method for producing such
materials.

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The term "hard tissue" used in this patent
application is used as a general term for tissues of
organisms including calcium, proteins, polysaccharides
and other trace elements such as bone and bone marrow
of mammals, birds and fishes, shells, crustacean
integument, egg shells and corals.

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Background of the invention

Various kinds of functional foods containing
mainly calcium, which may be used as supplemental
foods for osteoporosis or other bone-related diseases,
are on the market. Currently, these calcium
25 containing foods are mainly made from calcium
carbonate, calcium lactate, calcium phosphate, or
cattle bone powder, egg shells or fish bone powder
(natural bone-derived calcium).

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Although the relationship between the
characteristics of calcium components ingested to the
body and its degradation and intake inside the body is
not completely solved, it is considered in general
that intake of natural bone-derived calcium is

1 distinctly superior to simple intake of calcium salts
in terms of quick recovery from bone diseases.
However, there is a disadvantage in the commercially
available natural calcium based foods such as cattle
5 bone powder or fish bone powder in their problematic
ingestibility. That is, they are not fully degraded
by gastric acid due to their coarse particles since
they are made into powder by grinding.

A method was proposed in which mammal bones
10 are decalcified using acidic solution in order to
achieve efficient intake of calcium components
(Japanese Laid-Open Patent Application No. 4-16165).
This method basically provides calcium intake
supplements or calcium replenishments in which soluble
15 calcium is added to an acid soluble fraction obtained
by decalcifying ground bone powder using an aqueous
solution of inorganic acid such as hydrochloric acid,
sulfuric acid and nitric acid or that of organic acid
such as formic acid and acetic acid, or to a water
20 soluble fraction obtained by adding an enzyme to the
above fraction and hydrolyzing it, or to a fraction
obtained by dialyzing the above fractions to remove
bone-derived calcium salts.

More specifically, ground bone is suspended
25 in an aqueous solution of pH 2-3 containing the above-
mentioned inorganic acid or organic acid and the bone
is decalcified by stirring for 30 min. to a few hours.
Then protease (e.g. pepsin) is added to carry out
enzymatic degradation and a resulting acid soluble
30 fraction or water soluble fraction of molecular weight
3000 to 60000 Da is used as the calcium intake
supplements, or the above fractions are further
desalinized to produce fractions comprising a mixture

1 of peptides and proteins and soluble calcium lactate
or calcium carbonate is added thereto to produce the
calcium replenishments.

On the other hand, mammal bones contain not
5 only calcium but also physiologically active
substances such as collagen proteins including
peptides or polypeptides, amino acids, proteoglycan
and trace amounts of bioactive elements. It is known
that these substances other than calcium are also
10 desirable supplements for bone related diseases from
the point of view of enhancing physiological activity
(hormone balance or activity of enterobacteria) which
is very important in biological systems. Especially,
it has been found that proteins such as BMP or BSP-II
15 discovered recently are deeply involved with the
formation of bone. Their use as pharmaceuticals has
been studied.

In the meantime, since the above mentioned
conventional method used strong acids to decalcify
20 bone components, the physiological activity of
components contained in products are lowered. Also,
since physiologically active substances such as
proteins and proteoglycan are denatured by heat
generated during a grinding process of mammal bones,
25 which is carried out before the decalcification
process, the products do not contain natural
components of the bone.

Summary of the invention

30 Therefore, the object of the present
invention is to provide hard tissue intactly dissolved
materials having an excellent effect on bone-related
diseases and a method for producing the materials in

1 which hard tissues are completely dissolved in a short
amount of time without denaturing the original
components.

5 To achieve the object, according to the
present invention, ground hard tissues and edible
acids are mixed and dissolved under reduced pressure.
The hard tissues are mainly bones of mammals, birds
and fishes, however, bone marrow or teeth or shells,
crustacean integument and corals may also be utilized.
10 Moreover, it is preferable to use lactic acid as the
edible acid.

 The completely dissolved hard tissues
according to the present invention may be obtained by
mixing (immersing, dissolving, suspending) bone
15 tissues with acidic solution and dissolving them under
reduced pressure. When this mixed solution is stirred
in a state of reduced pressure, e.g., under vacuum,
air present in fine pores of bone powder is degassed
and the acidic solution goes into the pores.

20 When the bone powder is mixed and immersed
in the edible acid under standard atmospheric
pressure, a rapid and sufficient dissolution cannot be
achieved since the solution cannot contact the bone
due to generation of a bubbling phenomenon. However,
25 anti-bubbling effect is increased under reduced
pressure and the contact between the bone and the
solution is enhanced so that the time required for the
dissolving process is shortened and the dissolving
efficiency is increased.

30 That is, in a state of removing bubbles
formed, the outer surface and inner fine pores of the
bone powder are completely contacted with the acidic
solution and dissolved. The absolute magnitude of the

1 acidic solution which contacts the inside of the bone
tissue under reduced pressure is of tens-fold to
hundreds-fold of the contact amount of the acidic
solution stirred under standard atmospheric pressure.
5 For this reason, time required for the dissolving
process is remarkably shortened and, more importantly,
it becomes possible to dissolve bone components
intactly (the relationship between the degree of
reduced pressure and the solubility is shown in
10 FIG.7). This is because the acidic solution makes
contact with the fine bone powder from the inside and
calcium, proteins, trace amount physiological active
substances, etc., contained in the bone tissues are
sufficiently extracted.

15 Since time required for the dissolving
process can be reduced, denaturing of bone components
due to the acidic solution is minimized as much as
possible and their yield is remarkably increased.

 The application of the hard tissue intactly
20 dissolved materials according to the present invention
is not limited to functional foods (especially,
calcium replenishment). The materials are also
applicable to pharmaceuticals, cosmetics, bone fillers
(medical materials), toothpaste materials, internal
25 pellets having sustained release property and so on.

 Note that when the materials are eaten by a
human as functional foods, it is necessary that they
are not poisonous to the human body. From this point
of view, strong acids such as hydrochloric acid cannot
30 be used in the present invention. According to
experiments conducted, use of organic acids such as
acetic acid (including the one from rice), wine
vinegar and formic acid is found to be preferable as

1 the soluble solution which is not poisonous to human beings.

Also, it is confirmed that since calcium components of hard tissues have been converted to
5 calcium lactate in the hard tissue intactly dissolved material obtained as a final product when lactic acid is used as the dissolving solution, intake of the material in a human body is very high, and the product has high physiological activity, in which all the
10 components of the bone (tissue) are kept intact.

Brief description of drawings

FIG.1 shows a chromatogram of an example of the hard tissue intactly dissolved materials according
15 to the present invention;

FIG.2 shows a chromatogram of decalcificated components of a mammal hard tissue obtained using a conventional method;

FIG.3 shows Western blotting indicating
20 existence of BMP in a hard tissue intactly dissolved material;

FIG.4 is a diagram constructed based on soft X-ray photograph taken after an experiment in which the hard tissue intactly dissolved materials are
25 implanted underneath the back region skin of a rat;

FIG.5 is a diagram showing measured values of alkaliphosphatase activity of the hard tissue intactly dissolved material;

FIG.6 shows Western blotting indicating an
30 existence of BSP-II in hard tissue intactly dissolved materials; and

FIG.7 is a graph showing the relationship between the degree of reduced pressure and the

1 solubility.

Preferable embodiments of the present invention

FIG.1 is an example of chromatograph showing
5 components of the hard tissue intactly dissolved
materials according to the present invention. As
shown, the molecular weight of organic matter
originally contained in the hard tissue may be
confirmed to be a minimum of hundreds Da to a maximum
10 of two million Da or more. However, using the
conventional method, organic matter in a small range,
from 3000 to 60000Da, can only be obtained as shown in
FIG.2. This indicates that components having high
physiological activity are lost during the
15 decalcification and desalinization processes. On the
other hand, according to the present invention, it is
possible to obtain organic matter such as proteins
(including peptides) and polysaccharides of molecular
weight between 100 and two million Da without losing
20 components having high physiological activities.
Embodiments

The hard tissue intactly dissolved materials
according to the present invention may be obtained,
for example, using the following procedure.

25 First, mammal bones or fish shells are
ground, while generation of heat is restrained as much
as possible, until their particle size becomes
appropriate. The ground particles are then washed
with water. It is preferable that the particle size
30 is no more than five mm, for instance. After washing,
the particles are mixed with the lactic acid solution
of appropriate concentration, e.g., 10 percent, and
the container containing the solution is placed in a

1 degassing apparatus and is left under reduced pressure
while the solution is stirred. In this case, as the
rate of degassing is increased, the rate of dissolving
process of the hard tissue is increased. Alteration
5 of the components due to the reduced pressure could
not be confirmed. Thus, in order to reduce the
process time as much as possible, it is necessary to
stir the solution in a state which is as close as
possible to a vacuum condition. Although the
10 temperature of stirring is not limited, it is
desirable to use a low temperature (for example, from
0 degree to 20 degrees), preferably about four
degrees, in order to prevent the growth of bacteria
and so on.

15 All the components of the mammal bones and
fish shells may be completely dissolved after five to
six hours of stirring under the above mentioned vacuum
condition. The process time may be significantly
reduced compared with the case in which no reduction
20 of pressure is carried out. Moreover, if reduction of
pressure is not carried out, not all the components of
the bone tissue can be dissolved. However, by
reducing the pressure, all the components can be
dissolved without denaturing.

25 The yield of calcium, organic matter and
trace elements contained originally in hard tissues is
significantly increased under the above-mentioned
reduced pressure condition (not necessarily a vacuum
condition) compared with the case in which no
30 reduction of pressure is carried out. It was found
that when 10% lactic acid solution and reduced
pressure condition (e.g. vacuum condition) are
employed, the above-mentioned yield is increased to

1 about six to ten-fold compared with the yield under
non-reduced pressure.

 Also, it is possible to obtain calcium,
organic matter and trace elements originally contained
5 in hard tissues substantially intact. That is,
although the molecular weight of proteins (including
peptides) contained in normal natural hard tissues
ranges from hundreds to two million Da as shown in
FIG.1, the molecular weight of hard tissue dissolved
10 materials obtained under non-reduced pressure
condition is merely in the range of 3000 to 60000 Da
as shown in FIG.2. On the other hand, it is possible
to obtain hard tissue components whose molecular
weight range from 100 to two million Da when the hard
15 tissue particles-lactic acid mixed solution is stirred
under reduced pressure.

 The hard tissue intactly dissolved materials
according to the present invention are materials in
which proteins are dissolved in non-denatured state
20 and the materials are considered to be including
proteins having sufficient physiological activities.
For this reason, existence or non-existence of BSP-II
(bone sialoprotein II) and BMP (bone morphogenetic
protein) in hard tissue intactly dissolved materials,
25 and physiological activity of BMP are investigated.

 FIG.3 shows a study on existing BMP in a
hard tissue intactly dissolved material. The Western
blotting method was used and the confirmation was made
using anti-bovine BMP-2 monoclonal antibody. As a
30 result, a band of about 24000 molecular weight which
responded to the anti-bovine BMP-2 monoclonal antibody
was detected in lane 2. On the other hand, the same
type of band was detected at the region of about 28000

1 molecular weight in lane 4. This indicates that BMP
existed in the hard tissue intactly dissolved
materials and that it existed in a more intact state
compared with the one from the guanidine extracted
5 method which is generally carried out.

Then, in order to determine if the protein
has a physiological activity, its bone forming ability
was examined by implanting the materials underneath
the skin along a back region of a rat.

10 FIG.4 is a diagram constructed based on soft
X-ray photograph taken after the experiment in which
the hard tissue intactly dissolved materials are
implanted underneath the skin along a back region of a
rat. Since opaque images are shown in 2 and 3, it is
15 evidenced that the hard tissue intactly dissolved
materials has bone forming ability.

FIG.5 shows results of measuring values of
alkaliphosphatase activities of pellets which were
calcificated. The values of 2 and 3 are obviously
20 higher than that of 1 which is a control and the
activity of 3 is a little higher than that of 2.
Therefore, it is considered that the opaque image
shown in FIG.4 was not due to simple deposits of
calcium and showed the bone inducing activity.

25 FIG.6 shows the examination of an existence
of BSP-II in hard tissue intactly dissolved materials.
The Western blotting method using the anti-bovine BSP-
II monoclonal antibody was employed. As shown in the
figures, bands which responded to the anti-bovine BSP-
30 II monoclonal antibody were detected in the hard
tissue intactly dissolved materials (in lanes 2 and
3).

As mentioned above, the existence of

1 proteins involved in bone formation such as BMP and
BSP-II, whose use in treatment of bone-related
diseases, e.g., osteoporosis have drawn attention
recently, in the hard tissue intactly dissolved
5 materials was confirmed. Thus the hard tissue
intactly dissolved materials according to the present
invention can provide not only the calcium salts
supplements but also various products which contribute
to recovery of bone-related diseases by supplying
10 proteins, which enhance the formation of bone,
together with other trace amount physiologically
active elements.

Also, when lactic acid and reduced pressure
condition are used, bone calcium may be obtained
15 mainly in the form of soluble calcium. When the
calcium is used as a functional food, its
digestibility is far more excellent than the other
calcium salts.

The hard tissue intactly dissolved materials
20 according to the present invention may be produced
without using the lactic acid solution. When milk
(dry milk) (and sugar if necessary) is added to the
hard tissue powder and lactic acid bacterium is
further added to it with stirring at a temperature
25 higher than room temperature, e.g., 37 degrees, the
milk is converted to a lactic acid solution and
dissolves the hard tissue powder. Thus, if the lactic
acid bacterium is added to milk and mixed with the
hard tissue powder under the reduced pressure
30 condition, it is possible to intactly dissolve the
natural hard tissue components in a short amount of
time similar to the above-mentioned embodiment. In
this case, it is preferable that the initial

1 temperature is high, however, if the pressure is
reduced when the milk is converted to the lactic acid
solution, the same result as the previous embodiment
may be obtained. Note that the process time becomes a
5 little longer if the above conditions are used due to
the time required for conversion to lactic acid.

Also, the hard tissue intactly dissolved
materials according to the present invention are not
limited to the ones obtained by the process described
10 in the above mentioned embodiment. For example, the
edible acid is not limited to lactic acid. The reason
that lactic acid is used in the above embodiment is to
obtain a dissolved material, which can be eaten with
least resistance in terms of taste and other factors,
15 on the assumption that the material is eaten directly
as a recovering agent from bone-related diseases or a
functional food. Thus, if dissolved materials which
are not poisonous to human being can be obtained, it
is not necessary to use the lactic acid as a
20 dissolving solution. If the same result can be
obtained, it is possible to use acetic acid, citric
acid, acidic juices, etc., as the dissolving solution.
Not to mention that, if the final product (the hard
tissue intactly dissolved materials) is not used as a
25 food material, it is possible to use non-edible acids
such as hydrochloric acid.

Although it is explained that the mixture of
the bone tissue powder and the lactic acid solution in
the container is stirred under reduced pressure
30 condition (e.g., vacuum condition) in the previous
embodiment, the meaning of using the reducing pressure
is to ease the contact of the acidic solution to the
interspace of a powder particle. Therefore, it is

1 preferable to increase the number of contacts between
the interspace of a hard tissue powder and the acidic
solution in order to enhance a dissolving effect.
Thus, it is possible to change the degree of reduced
5 pressure so that the acidic solution which goes into
interspace of fine pores can move. For example, after
30 minutes of stirring under vacuum, the pressure is
returned to atmospheric for five minutes and then back
to vacuum for 30 minutes, and this pattern is
10 repeated. The time of a pattern is not particularly
limited. It is acceptable if the pattern repeats
reduction and non-reduction of pressure by which the
dissolution of hard tissue powder is completed in a
short amount of time.

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Possible use in industry

Since the hard tissue intactly dissolved
materials according to the present invention includes
not only calcium but also bone forming proteins such
20 as BMP and BSP-II, in intact form, whose use in
treatment of bone-related diseases (osteoporosis) have
drawn attention recently, it is possible to provide
functional foods which have positive effects on bone
formation necessary for the bone-related diseases such
25 as osteoporosis. Moreover, when a lactic acid is used
as dissolved agent, since it is obtained in the form
of soluble calcium, its digestibility as a functional
food is far more excellent compared with other calcium
salts.

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CLAIMS

1. A hard tissue intactly dissolved material including organic matter containing at least proteins, calcium and trace elements, obtained by dissolving natural hard tissues, wherein said organic matter exist in a non-denatured state.

2. A hard tissue intactly dissolved material, wherein proteins obtained by dissolving natural hard tissues includes bone morphogenetic protein and bone sialoprotein II.

3. The hard tissue intactly dissolved material according to claim 1 further comprising soluble calcium.

4. Food materials, feed, pharmaceuticals and cosmetics including the hard tissue intactly dissolved material as claimed in claims 1 and 2.

5. A method for producing a hard tissue intactly dissolved material, wherein ground hard tissues and an edible acid are mixed and said mixture is dissolved under reduced pressure.

6. The method for producing a hard tissue intactly dissolved material as claimed in claim 5, wherein a dissolving temperature is between 0 and 20 degrees.

7. A method for producing a hard tissue intactly dissolved material, wherein hard tissue powder and lactic acid solution are mixed and said mixture is dissolved under reduced pressure.

8. The method for producing a hard tissue intactly dissolved material as claimed in claim 7, wherein said lactic acid solution is obtained by adding lactic acid bacterium to milk and stirring at a

This Sheet Has Been Replaced





1 temperature no less than room temperature.

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This Sheet Has Been Replaced



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