



PROCESS FOR EXTRACTING PROTEIN CURCULIN

BACKGROUND OF THE INVENTION

[Field of the Invention]

This invention relates to a process for producing protein curculin, which will be also called curculin in some cases hereinafter, of improved qualities.

[Description of the Prior Art]

The applicant formerly found out a process which comprised thoroughly washing Curculigo latifolia fruits with water until the washing water was not colored any longer and then extracting curculin by using an aqueous solution of a salt of a concentration of 0.01 M or above (refer to Japanese Patent Laid-Open No. 104263/1990).

However, this water-washing operation suffers from several problems such that it achieves only an insufficient effect of eliminating impurities and causes browning of the Curculigo latifolia fruits.

Further, the protein curculin extract obtained by the above-mentioned salt-extraction process contains the salt and thus has a saltiness, which makes it necessary to desalt the extract before applying to foods. Furthermore, there is a fear of bacterial spoilage of the protein curculin extract thus obtained, since it is neutral in nature. Furthermore, the



above-mentioned process achieves only an unsatisfactory extraction efficiency.

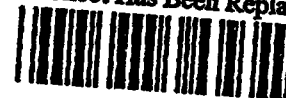
SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a process whereby protein curculin of improved qualities can be produced by treating Curculigo latifolia fruits in such a manner as to selectively eliminate impurities other than curculin without causing browning of the Curculigo latifolia fruits and then extracting curculin.

It is another object of the present invention to provide a process for extracting protein curculin whereby a curculin extract having no saltiness and being in little fear of spoilage can be obtained from Curculigo latifolia fruits or processed Curculigo latifolia fruits and the extraction efficiency can be elevated.

To achieve the above-mentioned objects, the present inventors have conducted extensive studies. As a result, they have successfully found out that impurities other than curculin can be selectively eliminated from plant bodies containing curculin under acidic conditions.

The present invention, which has been completed based on the above-mentioned finding, provides a proc-



ess for extracting protein curculin which comprises adding an aqueous acid solution to plant bodies containing protein curculin so as to adjust the pH of the resulting mixture to 3 or below and then extracting the protein curculin (hereinafter referred to as the first invention).

The present invention further provides a process for extracting protein curculin which comprises adding an aqueous acid solution to plant bodies containing protein curculin, treating the resulting mixture (1) under such conditions as to give a pH value of from 1.8 to 4.0 and then extracting the protein curculin from the mixture (2) thus treated (hereinafter referred to as the second invention). The term "the present invention" as used herein includes both of the first and second inventions.

According to the process for extracting protein curculin of the present invention, curculin-containing plant bodies are treated with an aqueous acid solution and, as a result, the efficiency of the elimination of impurities can be elevated compared with the conventional water-washing operation. Further, browning of the Curculigo latifolia fruits would not proceed, since the treatment is effected under acidic conditions. Thus, protein curculin having improved qualities,



compared with the conventional ones, can be obtained.

According to the process for extracting protein curculin of the present invention, further, a curculin extract having no saltiness and being in little fear of spoilage can be obtained from curculin-containing plant bodies and the extraction efficiency can be elevated. Furthermore, the curculin extract obtained by the process of the present invention has no unnecessary saltiness and is in little fear of spoilage in the extraction step, which makes it easily applicable to foods. Furthermore, the above-mentioned curculin extract can be easily subjected to post-extraction operations, for example, clarification by microfiltration or concentration by ultrafiltration, since the impurities present therein are liable to aggregate due to the acidity.

DETAILED DESCRIPTION OF THE INVENTION

First, the process for extracting protein curculin according to the first invention will be described in detail.

The curculin-containing plant bodies to be used in the first invention include Curculigo latifolia fruits and processed Curculigo latifolia fruits as well as plant cells obtained through biological techniques with the use of a gene originating in curculin. The plant



cells obtained through biological techniques with the use of a gene originating in curculin are not particularly restricted, so long as they are one which have been obtained by using a curculin gene or a curculin-like gene.

The above-mentioned Curculigo latifolia fruits or processed Curculigo latifolia fruits are as follows.

As the Curculigo latifolia fruits or processed Curculigo latifolia fruits, fresh Curculigo latifolia fruits and processed ones (for example, dried or frozen fruits, powders or grinds thereof, or sarcocarp puree) may be cited. It is preferable to use Curculigo latifolia fruits free from peels and seeds, since no curculin is contained in peels or seeds.

In the present invention, the method for drying the fruits is not particularly restricted. It is recommended, however, to conduct drying in the shade, cold-drying, freeze-drying, vacuum-drying or drying in a relatively low temperature range therefor.

In the present invention, the method for freezing the fruits is not particularly restricted. As the method therefore, slow freezing, rapid freezing and self-freezing may be cited.

In the present invention, the method for powdering or grinding the fruits and processed fruits is not

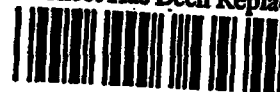


particularly restricted. It is undesirable, however, to powder or ground seeds distributed in the sarcocarp, since these seeds are rich in oily components and therefore make the subsequent purifying operation difficult when powdered or ground.

As the aqueous acid solution to be used in the first invention, those capable of giving a pH value of 3 or below when mixed with the curculin-containing plant bodies are usable. However, the curculin-containing plant bodies have a buffer capacity of neutralizing an acid. Thus a mere aqueous acid solution of a low concentration is insufficient for maintaining the acidity at the desired level and therefore an aqueous acid solution containing an acid in an increased amount sufficient for compensating for said buffer capacity should be used therefor.

In the first invention, it is preferable that the mixture of the curculin-containing plant bodies with the aqueous acid solution has a pH value of 2 or below, still preferably from 0.5 to 1.8. This is because the extraction efficiency can be thus elevated. Although the protein curculin can be extracted when the pH value of said mixture is 0.5 or below, it is feared in this case that the protein curculin might be denatured.

As the method for measuring the pH value of the



above-mentioned mixture, publicly known ones, for example, the method with the use of a glass electrode and the one based on the comparison of changes in pH test paper sheets may be cited. In the present invention, the logarithm of the reciprocal of the hydrogen ion activity (H^+) is defined as pH. Since this value corresponds to the value measured with a common glass electrode pH meter, the pH value may be measured in the present invention by using a common glass electrode pH meter.

As the acid to be used in the extraction, sulfuric acid, hydrochloric acid, nitric acid and phosphoric acid are preferable. In addition, sulfur oxides such as sulfonic acid, halogen oxides such as perchloric acid, chloric acid, chlorous acid and hypochlorous acid, nitrogen oxides, various phosphorus oxides such as orthophosphoric acid and pyrophosphoric acid, acids constituting the TCA cycle, vitamins such as ascorbic acid, dibasic acids such as oxalic acid, various other organic acids and mixtures thereof may be used therefor.

It is preferable to use strong acids such as sulfuric, hydrochloric and nitric acids, since the amount of the employed acid can be reduced thereby. In particular, sulfuric acid is the most desirable there-



for from the viewpoint of the handling characteristics of not corroding a stainless container. Further, phosphoric acid is preferable from the viewpoints of safety and flavor, though it is to be used in an amount about 5 to 20 times as much as a strong acid in order to give a pH value of 3 or below.

The acid concentration of the above-mentioned aqueous acid solution may be preferably 0.01 N or above, still preferably from 0.02 to 1.0 N, in the case where sulfuric acid is used as the acid; 0.005 N or above, still preferably from 0.02 to 1.0 N, in the case where nitric acid is used as the acid; 0.005 N or above, still preferably from 0.02 to 1.0 N, in the case where hydrochloric acid is used as the acid; or 0.03 N or above, still preferably from 0.1 to 5.0 N, in the case where phosphoric acid is used as the acid.

Next, a typical example of the process for extracting protein curculin by using the above-mentioned aqueous acid solution according to the first invention will be given.

Protein curculin can be extracted by adding an aqueous acid solution directly to curculin-containing plant bodies in such a manner as to adjust the pH value of the resulting mixture to 3 or below and stirring or homogenizing the mixture followed by, for example,



filtering or centrifuging. The extraction may be effected over such a period of time as to allow the plant bodies to be sufficiently suspended in the aqueous acid solution. The extraction time preferably ranges from 1 minute to 2 hours. The extraction may be carried out at room temperature.

A preferable process comprises, prior to the extraction with the aqueous acid solution, adding water to the above-mentioned plant bodies, stirring or homogenizing the resulting mixture, repeatedly filtering or centrifuging it to thereby thoroughly wash pulp components with water and then extracting curculin with the above-mentioned aqueous acid solution from the pulp components thus obtained. This is because water-soluble coloring matters and other impurities can be eliminated from the above-mentioned plant bodies by washing the same with water prior to extracting with the aqueous acid solution, since protein curculin is not extracted with just water.

The protein curculin extract obtained by extracting with the aqueous acid solution is subjected to post-treatments such as concentration, neutralization, desalting or drying and thus a protein curculin-containing product which is sufficiently usable in practice can be obtained. It is particularly prefera-



ble to neutralize the above-mentioned protein curculin extract. This is because protein curculin, which has a low solubility in a neutral aqueous solution, can be precipitated by neutralizing and the post-treatments such as drying can be easily performed by collecting the precipitates thus formed by, for example, centrifuging or filtering.

The protein curculin extract may be neutralized by appropriately adding, for example, sodium hydroxide, potassium hydroxide, calcium hydroxide or magnesium hydroxide.

In the present invention, the method for concentrating the protein curculin extract is not particularly restricted. Thus it may be performed with the use of, for example, an ultrafiltration membrane.

In the present invention, the method for the desalting is not particularly restricted. Thus it may be effected with the use of for example, a dialysis membrane, an ultrafiltration membrane or a desalting machine.

In the present invention, the drying may be effected in the same manner as those cited above for drying the Curculigo latifolia fruits.

After the neutralization, the protein curculin extract obtained by extracting with the above-mentioned



aqueous acid solution may be further purified by a publicly known method for purifying proteins, for example, salting out, solvent precipitation or chromatography, if necessary.

The above-mentioned salting out may be effected by using, for example, ammonium sulfate, sodium sulfate, potassium phosphate, magnesium sulfate, sodium citrate or sodium chloride.

The above-mentioned solvent precipitation may be effected by appropriately adding a polar solvent such as methanol, ethanol, isopropanol or acetone.

The above-mentioned chromatographic purification may be effected by ion exchange chromatography with the use of, for example, CM-Sepharose or Amberlite IRC-50 or gel filtration chromatography with the use of, for example, Sephadex G-100.

Next, the process for extracting protein curculin of the second invention will be described in detail.

As a method for extracting curculin from the above-mentioned mixture (2), a method wherein the pH of the mixture (2) is adjusted to 3 or below followed by extracting and another one wherein it is extracted by using an aqueous solution of a salt of a concentration of 0.01 M or above may be cited.

The aqueous acid solution to be used for preparing



the mixture (1) is not particularly restricted and various ones including the aqueous acid solutions employed in the extraction step in the first invention are usable therefor. However, the curculin-containing plant bodies have a buffer capacity of the neutralizing an acid. Thus a mere aqueous acid solution of a low concentration is insufficient for maintaining the acidity at the desired level and therefore an aqueous acid solution containing an acid in an increased amount sufficient for compensating for said buffer capacity should be used therefor.

It is not preferable that the pH value of the mixture (1) is lower than 1.8, since a large amount of curculin would be extracted into the treated solution in this case. Although curculin is extracted at pH 3.0 or below, the amount of the curculin thus extracted is very small. Therefore it is preferable to effect the treatment at pH 1.8 to 4.0, since the effect of eliminating hardly removable impurities thus achieved is more advantageous.

On the other hand, it is not preferable that the pH value of the mixture (1) exceeds 4.0, since impurities are scarcely eliminated and the browning of the Curculigo latifolia fruits proceeds in this case.

It is particularly preferable that the pH value of



the mixture (1) is adjusted to 2.0 to 3.0.

Next, a typical example of the embodiment of the process for extracting protein curculin of the second invention will be described.

To curculin-containing plant bodies is added an aqueous acid solution to thereby adjust the pH value of the resulting mixture (1) to 2.0 to 3.0. After stirring or homogenizing, the treated solution is separated by, for example, filtering or centrifuging. Although impurities can be effectively eliminated by effecting a series of the operations (treatments) once, it is preferable to repeat these operations several times so as to sufficiently eliminate the impurities.

From the obtained residue of the curculin-containing plant bodies which have been thus treated with the aqueous acid solution [the mixture (2)], curculin can be extracted by a method with the use of an aqueous solution of a salt of a concentration of 0.01 M or above or by another method of adjusting the pH value of the mixture (2) to 3 or below.

Next, the above-mentioned method with the use of an aqueous solution of a salt of a concentration of 0.01 M or above will be described.

Examples of the salt usable in the extraction include hydrochlorides of sodium, potassium, calcium,



magnesium and ammonium, phosphates of sodium, potassium, calcium, magnesium and ammonium, carbonates of sodium, potassium, calcium, magnesium and ammonium, sulfates and sulfites of sodium, potassium, calcium, magnesium and ammonium, nitrates and nitrites of sodium and potassium, lactates of sodium and calcium, alum, burnt alum, sodium acetate, pyrophosphates of sodium and potassium propionates of sodium and calcium, sodium benzoate, sodium fumarate and sodium polyacrylate.

The concentration of the aqueous salt solution may be at least 0.01 M. It preferably ranges from 0.1 to 1.0 M, from the viewpoint of the purification step.

Now, an example of the above-mentioned extraction with an aqueous salt solution will be given.

To the residue of the curculin-containing plant bodies [i.e., the mixture (2)], from which impurities have been eliminated by the above-mentioned treatment, is added an aqueous solution of sodium chloride of a concentration as specified above. After homogenizing, the obtained mixture was extracted by centrifuging or filtering. The salt-extract of curculin thus obtained may be purified by conventional procedures such as concentration, desalting, salting out or chromatography.

In the present invention, the method for concen-



tration is not particularly restricted. Thus it may be effected with the use of, for example, an ultrafiltration membrane.

In the present invention, the method for desalting is not particularly restricted. Thus it may be effected by molecular sieve chromatography or with the use of, for example, a dialysis membrane, an ultrafiltration membrane or a desalting machine. The desalting may be preferably continued at least until the curculin-containing component shows no saltiness.

The purification by salting out or chromatography can be effected in the same manner as those described above regarding the first invention.

Also, the pH value of the above-mentioned mixture (2) may be adjusted to 3 or below by the same method as the one described above regarding the first invention.

The protein curculin-containing product obtained in accordance with the present invention (the first and second inventions) may be taken as a taste-modifier or a sweet protein either as such or after neutralizing. Alternately, it may be appropriately blended into foods, drinks or drugs.

The content of protein curculin in the above-mentioned taste-modifier or sweet protein may be determined depending on the purpose and usage by reference



to the taste modification activity and sweetness activity of the protein. The content of highly pure curcumin may preferably range from 0.1 to 1,000 ppm, still preferably from 1 to 100 ppm. The term "highly pure curcumin" as used herein means a product obtained by, when extracted with an aqueous salt solution, effecting salting out followed by ion exchange chromatography, gel filtration chromatography and salting out, or when extracted with an aqueous acid solution, neutralizing followed by ion exchange chromatography, gel filtration chromatography and salting out.

The taste-modifier containing the protein curcumin obtained by the present invention may be formulated into, for example, a powder, a solution, a sheet, a spray or an emulsion.

The contents of the protein curcumin in the above-mentioned foods, drinks and drugs may be determined depending on the purpose and usage by reference to the taste modification activity and sweetness activity of the protein curcumin. More particularly, the contents of the highly pure curcumin in foods, drinks and drugs may preferably range from 1 to 1,000 ppm, still preferably from 1 to 100 ppm. The foods, drinks and drugs containing the protein curcumin obtained by the present invention may be processed into various



forms depending on the properties.

When the protein curculin obtained by the present invention is held in the mouth and then a sour substance or water is taken, or it is taken together with a sour substance or water, it acts on the receptor membrane of the tongue and thus exerts an effect of giving a sweetness. When the protein curculin obtained by the present invention or, for example, a food containing the same is previously taken, a sweetness is felt at the intake of a sour substance or water.

The taste-modification activity of protein curculin may be measured in the following manner. 3 ml of a protein curculin solution (in a 50 mM phosphate buffer solution, pH 6.0) is held in the mouth for 3 minutes, followed by slight rinsing out of the mouth. Next, 3 ml of a 0.1 M solution of citric acid is taken and the intensity of the sweetness thus felt is expressed in terms of the sweetness of sucrose (molar concentration of sucrose) and employed as the taste-modification activity (sweetness intensity).

The following Table 1 shows the results of the measurement of the taste-modification activity (sweetness intensity) by using the highly pure curculin according to the present invention obtained in the Production Example 2 as will be given hereinafter.



[Table 1]

Curculin concn. ($\mu\text{g/ml}$)	14	28	56	111	222
Sweetness intensity (M)	0.08	0.17	0.24	0.30	0.36

To further illustrate the present invention in greater detail, the following Examples will be given.

Example 1

To 1 kg of freeze-dried Curculigo latifolia fruits was added 40 l of water. Then the mixture was subjected to solid-liquid separation with the use of a continuous centrifuge H130B (Kokusan Enshinki K.K.) under stirring in a mixture. After removing the liquid phase, 40 l of water was added again to the solid phase. Then the solid-liquid separation was repeated thrice in total until the liquid phase was not colored any longer.

Subsequently, 20 l of water was added to the solid phase thus obtained under stirring in a mixer. Then the pH of the mixture was adjusted to 1.5 by slowly adding a 30% solution of sulfuric acid. Next, the mixture was subjected to solid-liquid separation again with the use of a continuous centrifuge under stirring in a mixer. This extraction operation was repeated thrice to thereby give a crude curculin extract (liquid phase).



The concentration of curculin in this crude curculin extract was determined by the method as will be specified below. It was found that the curculin concentrations of the first, second and third extracts were respectively 149 $\mu\text{g/ml}$ (20.1 ℓ ; the amount of the extract, the same will apply hereinafter), 48 $\mu\text{g/ml}$ (19.7 ℓ) and 6 $\mu\text{g/ml}$ (19.9 ℓ).

Method for determining protein curculin:

10- μl portions of highly pure curculin (prepared in the following Production Example 2) solutions, the concentrations of which had been adjusted to 0.5 mg/ml, 0.2 mg/ml, 0.1 mg/ml, 0.05 mg/ml, 0.02 mg/ml and 0.01 mg/ml for the comparison of the intensities of bands, were electrophoresed on a polyacrylamide gel in accordance with the method of Laemmli [refer to Laemmli, U.K., Nature, 227, 680 (1970)].

After the completion of the electrophoresis, the polyacrylamide gel was stained with Coomassie Brilliant Blue R-250. After destaining, the color intensity of each sample was compared with the color intensities of highly pure curculin bands of known concentrations by using a densitometer. Thus the curculin contained in the sample solution was determined.

Production Example 1

The 3 aliquots of the crude curculin extract



obtained in the above Example 1 were combined and clarified (filtered) by using a ceramic membrane (Cefilt MF mfd. by NGK Insulators, Ltd.; nominal pore size: 0.1 μ m, form: monolithic, size: 30/4 in diameter - 19 pores x 1000 L x 2). The treatment time was 60 minutes.

The clarified extract thus obtained was concentrated to 4.2 ℓ by ultrafiltering with the use of a ceramic membrane (Cefilt UF mfd. by NGK Insulators, Ltd.; nominal pore size: 100 A, form: monolithic, size: 30/4 in diameter - 19 pores x 1000 L x 2). The treatment time was 90 minutes.

Next, the obtained concentrate was neutralizing by adjusting the pH to 7.0 by using sodium hydroxide. When tasted, this neutralizer concentrate showed neither any sourness nor saltiness but a refreshing sweetness of curculin.

Further, the precipitates formed in the neutralized solution were collected by centrifuging at 12,000 rpm for 10 minutes and dried. Thus about 5.2 g of a crude curculin powder was obtained.

Production Example 2

30 ml of the neutralized solution obtained in the above Production Example 1 was passed through a CM-Sephadex CL-6B column (22 mm in diameter x 18 cm in



height, column bed volume: 68 ml, mfd. by Pharmacia LKB Biotechnology) and adsorbed thereby. Subsequently, the unadsorbed fraction was washed with a 0.01 M phosphate buffer solution (pH 6.8) and the protein curculin was eluted with a solution containing a linear gradient of 0 to 1.0 M of sodium chloride (flow rate: 5 ml/hr., fraction volume: 5 ml, total amount of the eluate: 500 ml).

The protein thus eluted was monitored by a peak at 280 nm. It was found that a fraction containing the protein curculin was eluted at a sodium chloride concentration of around 0.4 M.

To this protein curculin-containing fraction was added ammonium sulfate in such a manner as to achieve 80% saturation and thus the active substance was precipitated (salting out). After centrifuging (32,000 rpm, 60 minutes), the precipitate thus obtained was dissolved in 1.5 ml of a 0.01 M phosphate buffer solution (pH 6.8) and concentrated. This concentrate was further separated with a 0.01 M phosphate buffer solution (pH 6.8) containing 0.5 M NaCl by using Sephadex (G-100 column, 1.6 cm in diameter x 58 cm, 160 ml, mfd. by Pharmacia LKB Biotechnology) (flow rate: 8.4 ml/hr., fraction volume: 2.8 ml, total amount of eluate: 182 ml; gel filtration).



The protein thus eluted was monitored by a peak at 280 nm. It was found that a peak of the protein curculin was eluted.

To the fraction of this peak was added ammonium sulfate in such a manner as to achieve 80% saturation and thus the active substance was precipitated (salting out). After centrifuging (32,000 rpm, 60 minutes), the precipitate thus obtained was dissolved in 1.5 ml of a 2 M solution of ammonium acetate. The resulting solution was treated with a Sephadex G-25 column (9.1 ml) which had been equilibrated with a 2 M solution of ammonium acetate (gel filtration) and then freeze-dried. Thus 8.6 mg of a powder of highly pure curculin was obtained.

Production Example 3

The procedure of Production Example 2 was repeated except that the CM-Sepharose CL-6B column employed in Production Example 2 was replaced with Amberlite IRC-50 (10 mm in diameter x 13 cm in height, column bed volume: 10 ml, mfd. by Organo) which had been equilibrated with a 0.05 M phosphate buffer solution (pH 5.5). Thus 8.9 mg of a powder of highly pure curculin was obtained.

Example 2 (extraction with sulfuric acid)

30 mg of freeze-dried Curculigo latifolia fruits



were weighed into a micro-centrifuge tube (1.5 ml in capacity). After adding 600 μ l of water thereto, the obtained mixture was stirred in a mixer for 1 minute and then centrifuged at 12,000 rpm for 10 minutes. Then the supernatant was removed and 600 μ l of water was added again to the residue. Then the procedure of removing the supernatant was repeated 4 times in total until the supernatant was not colored any longer.

To the obtained residue was added 600 μ l of a 0.0625 N solution of sulfuric acid (pH value of the mixture: 1.56). The resulting mixture was stirred in a mixer for 1 minute and then centrifuged at 12,000 rpm for 10 minutes to thereby obtain a crude curculin extract as the supernatant.

The concentration of curculin contained in this crude curculin extract was 154 μ g/ml (the curculin concentration was determined in the same manner as the one employed in Example 1).

Examples 3 to 8 and Comparative Examples 1 to 2
(Extraction with sulfuric acid)

The procedure of Example 2 was repeated except that 600 μ l of the 0.0625 N solution of sulfuric acid employed in Example 2 was replaced with 600 μ l of a 0.0039 N solution of sulfuric acid (Comparative Example 1) and 600 μ l of a 0.0078 N solution of sulfuric acid



(Comparative Example 2)(in these cases, the pH value of each mixture of the freeze-dried Curculigo latifolia fruits with the sulfuric acid solution was higher than 3) and 600 μ l of a 0.0156 N solution of sulfuric acid (Example 3), 600 μ l of a 0.0313 N solution of sulfuric acid (Example 4), 600 μ l of a 0.125 N solution of sulfuric acid (Example 5), 600 μ l of a 0.25 N solution of sulfuric acid (Example 6), 600 μ l of a 0.5 N solution of sulfuric acid (Example 7) and 600 μ l of a 1 N solution of sulfuric acid (Example 8)(in these cases, the pH value of each mixture of the freeze-dried Curculigo latifolia fruits with the sulfuric acid solution was lower than 3).

The following Table 2 shows the curcumin concentration of each crude curcumin extract and the pH value of each mixture.

The results given in Table 2 indicate that curcumin was extracted when the pH value of the mixture was 3.0 or below and the extraction efficiency of curcumin was further elevated at a pH value of 2.0 or below.

[Table 2]

Extraction with sulfuric acid	Sulfuric acid concentration (N)	pH of mixture	Curculin concentration (µg/ml)
Comp. Ex. 1	0.0039	3.57	—
2	0.0078	3.04	—
Ex. 3	0.0156	2.33	9
4	0.0313	1.89	130
5	0.125	1.25	144
6	0.25	0.98	153
7	0.5	0.73	144
8	1.0	0.55	157

Examples 9 to 16 and Comparative Example 3 (extraction with nitric acid)

The procedure of Example 2 was repeated except that 600 µl of the 0.0625 N solution of sulfuric acid employed in Example 2 was replaced with 600 µl of a 0.0039 N solution of nitric acid (Comparative Example 3) (in this case, the pH value of the mixture of the freeze-dried Curculigo latifolia fruits with the nitric acid solution was higher than 3) and 600 µl of a 0.0078 N solution of nitric acid (Example 9), 600 µl of a 0.0156 N solution of nitric acid (Example 10), 600 µl of a 0.0313 N solution of nitric acid (Example 11), 600 µl of a 0.0625 N solution of nitric acid (Example 12), 600 µl of a 0.125 N solution of nitric acid (Example 13), 600 µl of a 0.25 N solution of nitric acid (Example 14), 600 µl of a 0.5 N solution of nitric acid (Example 15) and 600 µl of a 1 N solution of nitric





acid (Example 16)(in these cases, the pH value of each mixture of the freeze-dried Curculigo latifolia fruits with the nitric acid solution was lower than 3).

The following Table 3 shows the curcumin concentration of each crude curcumin extract and the pH value of each mixture.

The results given in Table 3 indicate that curcumin was extracted when the pH value of the mixture was 3.0 or below and the extraction efficiency of curcumin was further elevated at a pH value of 2.0 or below.

[Table 3]

Extraction with nitric acid	Nitric acid concentration (N)	pH of mixture	Curcumin concentration (µg/ml)
Comp. Ex. 3	0.0039	3.49	—
Ex. 9	0.0078	2.81	3
10	0.0156	2.24	12
11	0.0313	1.85	129
12	0.0625	1.37	150
13	0.125	1.05	141
14	0.25	0.76	156
15	0.5	0.49	142
16	1.0	0.30	154

Examples 17 to 24 and Comparative Example 4
(extraction with hydrochloric acid)

The procedure of Example 2 was repeated except



that 600 μ l of the 0.0625 N solution of sulfuric acid employed in Example 2 was replaced with 600 μ l of a 0.0039 N solution of hydrochloric acid (Comparative Example 4)(in this case, the pH value of the mixture of the freeze-dried Curculigo latifolia fruits with the hydrochloric acid solution was higher than 3) and 600 μ l of a 0.0078 N solution of hydrochloric acid (Example 17), 600 μ l of a 0.0156 N solution of hydrochloric acid (Example 18), 600 μ l of a 0.0313 N solution of hydrochloric acid (Example 19), 600 μ l of a 0.0625 N solution of hydrochloric acid (Example 20), 600 μ l of a 0.125 N solution of hydrochloric acid (Example 21), 600 μ l of a 0.25 N solution of hydrochloric acid (Example 22), 600 μ l of a 0.5 N solution of hydrochloric acid (Example 23) and 600 μ l of a 1 N solution of hydrochloric acid (Example 24)(in these cases, the pH value of each mixture of the freeze-dried Curculigo latifolia fruits with the hydrochloric acid solution was lower than 3).

The following Table 4 shows the curculin concentration of each crude curculin extract and the pH value of each mixture.

The results given in Table 4 indicate that curculin was extracted when the pH value of the mixture was 3.0 or below and the extraction efficiency of curculin



was further elevated at a pH value of 2.0 or below.

[Table 4]

Extraction with hydrochloric acid	Hydrochloric acid concentration (N)	pH of mixture	Curculin concentration (µg/ml)
Comp. Ex. 4	0.0039	3.42	—
Ex. 17	0.0078	2.77	5
18	0.0156	2.19	14
19	0.0313	1.80	120
20	0.0625	1.34	149
21	0.125	1.01	155
22	0.25	0.73	144
23	0.5	0.45	156
24	1.0	0.28	151

Examples 25 to 31 and Comparative Examples 5 and 6

(extraction with phosphoric acid)

The procedure of Example 2 was repeated except that 600 µl of the 0.0625 N solution of sulfuric acid employed in Example 2 was replaced with 600 µl of a 0.012 N solution of phosphoric acid (Comparative Example 5) and 600 µl of a 0.023 N solution of phosphoric acid (Comparative Example 6) (in these cases, the pH value of each mixture of the freeze-dried Curculigo latifolia fruits with the phosphoric acid solution was higher than 3) and 600 µl of a 0.047 N solution of



phosphoric acid (Example 25), 600 μ l of a 0.094 N solution of phosphoric acid (Example 26), 600 μ l of a 0.188 N solution of phosphoric acid (Example 27), 600 μ l of a 0.375 N solution of phosphoric acid (Example 28), 600 μ l of a 0.75 N solution of phosphoric acid (Example 29), 600 μ l of a 1.5 N solution of phosphoric acid (Example 30) and 600 μ l of a 3N solution of phosphoric acid (Example 31)(in these cases, the pH value of each mixture of the freeze-dried Curculigo latifolia fruits with the phosphoric acid solution was lower than 3).

The following Table 5 shows the curcumin concentration of each crude curcumin extract and the pH value of each mixture.

The results given in Table 5 indicate that curcumin was extracted when the pH value of the mixture was 3.0 or below and the extraction efficiency of curcumin was further elevated at a pH value of 2.0 or below.

[Table 5]

Extraction with phosphoric acid	Phosphoric acid concentration (N)	pH of mixture	Curcumin concentration (μ g/ml)
Comp. Ex. 5	0.012	3.50	—
6	0.023	3.01	—



Ex. 25	0.047	2.45	7
26	0.094	2.13	15
27	0.188	1.84	128
28	0.375	1.61	138
29	0.75	1.43	143
30	1.5	1.21	146
31	3.0	0.97	147

Comparative Example 7 (extraction with acid solution of pH value higher than 3)

The procedure of Example 2 was repeated except that 600 μ l of the 0.0625 N solution of sulfuric acid employed in Example 2 was replaced with 600 μ l of a 2 N solution of acetic acid. It was found that the pH value of the mixture of the freeze-dried Curculigo latifolia fruits with the acetic acid solution was 3.54 and no curculin was detected in the crude extract.

Referential Example 1

The procedure of Example 1 was repeated except that the adjustment of the pH of the mixture to 1.5 with the use of 30% sulfuric acid was replaced with the addition of 3% by weight of common salt to thereby give a salt-extract of crude curculin.

The curculin concentrations of the first, second and third crude curculin salt-extracts were respectively 120 μ g/ml (20.0 ℓ ; the amount of the extract, the same will apply hereinafter), 42 μ g/ml (19.9 ℓ) and 20



µg/ml (19.8 μ) and each extract showed a pH value of 3 or above.

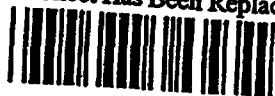
Referential Example 2

The 3 aliquots of the crude curculin extract obtained in Referential Example 3 were combined, clarified and concentrated in the same manner as the one described in Production Example 1. The clarification and concentration times were respectively 80 and 110 minutes. After neutralizing, no curculin precipitate was formed.

When the obtained concentrate was tasted, an intense saltiness was felt while a sweetness was also felt at the same time.

Referential Example 3

To salt out 15 ml of the concentrate obtained in the above Referential Example 2 instead of 15 ml of the neutralized solution employed in Production Example 1, ammonium sulfate was added in such a manner as to achieve 80% saturation and the precipitates thus formed were collected by centrifuging. The collected precipitates were dissolved in 15 ml of a 50 mM phosphate buffer solution (pH 6.0) and subsequently treated in the same manner as the one described in Production Example 1. Thus 7.3 mg of a powder of highly pure curculin was obtained.



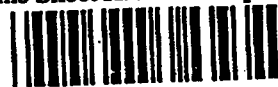
Referential Example 4

600 μ l of the 0.0625 N solution of sulfuric acid employed in Example 2 was replaced with 600 μ l of a 0.5 N solution of sodium chloride. It was found that the extract had a pH value of 3.44 and a curculin concentration of 117 μ g/ml.

Example 32 (treatment with sulfuric acid)

20 g of freeze-dried Curculigo latifolia fruits were weighed into a breaker and 800 ml of 0.0156 N sulfuric acid was added thereto, followed by stirring. The suspension thus obtained was centrifuged at 12,000 rpm for 10 minutes and thus separated into a supernatant (treated solution 1) and a precipitate (pulp fraction).

To the pulp fraction obtained above was added 800 ml of 0.0156 N sulfuric acid again. Then the mixture was stirred and centrifuged at 12,000 rpm for 10 minutes to thereby separate into a supernatant (treated solution 2) and a precipitate (pulp fraction). Further, 800 ml of 0.0156 N sulfuric acid was added to the pulp fraction again and the resulting mixture was stirred and centrifuged at 12,000 rpm for 10 minutes to thereby separate into a supernatant (treated solution 3) and a precipitate (pulp fraction). The treated solutions 1 to 3 thus obtained were subjected to poly-



crylamide gel electrophoresis (hereinafter referred to as SDS-PAGE) in accordance with the method of Laemmli [Laemmli, U.K., Nature, 227, 680 (1970)].

After the completion of the SDS-PAGE, the gel was stained with Coomassie Brilliant Blue R-250. After decoloring, the color intensity of each sample was compared with the color intensities of highly pure curculin bands of known concentrations by using a densitometer. Thus the curculin contained in the sample solution was determined. Impurities were confirmed depending on the color intensities of unknown bands observed on the polyacrylamide gel.

The following Table 6 shows the results of the analysis.

[Table 6]

Treated solution No.	pH	Curculin concn. ($\mu\text{g/ml}$)	Impurities
1	3.43	ND	broad band: <8 kd.
2	2.45	1	broad band: <8 kd. band: 12 kd.
3	2.08	8	ND

ND: not detected.

As the above Table 6 shows, impurities of 8 kd and 12 kd in molecular weight were extracted into the treated solutions. However, curculin was not detected



in the treated solutions 1 and 2 and 1 $\mu\text{g/ml}$ of curcumin was extracted in the treated solution 3. the precipitate (pulp fraction) suffered from little browning. Comparative Example 8 (treatment with water)

The procedure of Example 32 was repeated except that 800 ml of the 0.0156 N sulfuric acid employed in Example 32 was replaced with 800 ml of water. The following Table 7 shows the results.

[Table 7]

Treated solution No.	pH	Curcumin concn. ($\mu\text{g/ml}$)	Impurities
1	4.09	ND	slightly extracted: <8 kd.
2	4.45	ND	ND
3	4.93	ND	ND

ND: not detected.

As the above Table 7 shows, little impurities detectable by SDS-PAGE were extracted when treated with water. In this case, the precipitate (pulp fraction) suffered from browning.

Example 33 (extraction of curcumin with aqueous salt solution)

To the precipitate (pulp fraction) obtained in the above Example 32 was added 400 ml of a 0.5 M aqueous solution of NaCl, followed by stirring. The suspension thus obtained was centrifuged at 12,000 rpm for 10



minutes to thereby separate into a supernatant (extract 1) and a precipitate (pulp fraction). To the obtained pulp fraction was added 400 ml of a 0.5 M aqueous solution of NaCl again. The obtained mixture was stirred and centrifuged at 12,000 rpm for 10 minutes to thereby separate into a supernatant (extract 2) and a precipitate (pulp fraction). Furthermore, 400 ml of a 0.5 M aqueous solution of NaCl was added again to the obtained pulp fraction, followed by stirring. The obtained mixture was centrifuged at 12,000 rpm for 10 minutes to thereby separate into a supernatant (extract 3) and a precipitate (pulp fraction). The extracts 1 to 3 thus obtained were analyzed by SDS-PAGE in accordance with Laemmli's method as described in Example 32.

It was found that the curculin concentrations of the extracts 1 to 3 were respectively 114 ($\mu\text{g/ml}$), 43 ($\mu\text{g/ml}$) and 16 ($\mu\text{g/ml}$).

Comparative Example 9

The procedure of Example 33 was repeated except that the precipitate (pulp fraction) employed in Example 33 was replaced with the precipitate (pulp fraction) obtained in the above Comparative Example 8. It was found that the curculin concentrations of the extracts 1 to 3 thus obtained were respectively 108 ($\mu\text{g/ml}$), 39 ($\mu\text{g/ml}$) and 19 ($\mu\text{g/ml}$).



A comparison of the extracts of Example 33 with those of Comparative Example 9 indicates that the former ones suffered from less browning.

Example 34 (preparation of highly pure curculin)

To 140 ml of the extract 1 obtained in the above Example 33 was added ammonium sulfate so as to achieve 80% saturation and thus an active substance was precipitated. After centrifuging (32,000 rpm, 60 minutes), the precipitate thus formed was dissolved in 50 ml of a 0.01 M phosphate buffer solution (pH 6.8). Then it was passed through a CM-Sepharose CL-6B column (22 mm in diameter x 18 cm in height, 68 ml, mfd. by Pharmacia LKB Biotechnology) and adsorbed thereby.

After treating the unadsorbed fraction with a 0.01 M phosphate buffer solution (pH 6.8), curculin was eluted with a solution containing a linear gradient of 0 to 1.0 M of sodium chloride (flow rate: 5 ml/hr., fraction volume: 5 ml, total amount of the eluate: 500 ml). The protein thus eluted was monitored by a peak at 280 nm.

It was found that a fraction containing the taste-modifier curculin was eluted at a sodium chloride concentration of around 0.4 M.

To this protein curculin-containing fraction was added ammonium sulfate in such a manner as to achieve



80% saturation and thus the active substance was precipitated. After centrifuging (32,000 rpm, 60 minutes), the precipitate thus obtained was dissolved in 1.5 ml of a 0.01 M phosphate buffer solution (pH 6.8).

The concentrate was further separated with a 0.01 M phosphate buffer solution (pH 6.8) containing 0.5 M NaCl by using a Sephadex (mfd. by Pharmacia LKB Biotechnology) G-100 column (1.6 cm in diameter x 58 cm, 160 ml)(flow rate: 8.4 ml/hr., fraction volume: 2.8 ml, total amount of eluate: 182 ml).

The protein thus eluted was monitored by a peak at 280 nm. It was found that a peak of the taste-modifier curculin was eluted.

To the fraction of this peak was added ammonium sulfate in such a manner as to achieve 80% saturation. After centrifuging (32,000 rpm, 60 minutes), the precipitate thus obtained was dissolved in 1.5 ml of a 2 M solution of ammonium acetate.

The resulting solution was treated with a Sephadex G-25 column (9.1 ml) which had been equilibrated with a 2 M solution of ammonium acetate and then freeze-dried. Thus 8.5 mg of highly pure curculin was obtained.

Comparative Example 10

The procedure of Example 34 was repeated except



that 140 ml of the extract 1 employed in Example 34 was replaced with 100 ml of the extract 1 obtained in the above Comparative Example 9. Thus 8.0 mg of highly pure curculin was obtained.

Example 35 (extraction of curculin with aqueous acid solution)

The procedure of Example 33 was repeated except that 400 ml of the 0.5 M aqueous solution of NaCl employed in Example 33 was replaced with 400 ml of a 0.05 N sulfuric acid. It was found that the curculin concentrations of the extracts 1 to 3 were respectively 132 ($\mu\text{g/ml}$), 42 ($\mu\text{g/ml}$) and 10 ($\mu\text{g/ml}$) and the pH values of the extracts 1 to 3 were respectively 1.48, 1.45 and 1.44.

Example 36 (preparation of highly pure curculin)

As a substitute for 140 ml of the extract 1 employed in Example 34, 140 ml of the extract 1 obtained in the above Example 35 was used. The pH of the extract was adjusted to 6.0 with 1 N NaOH and adsorbed by a CM-Sepharose CL-6B column (22 mm in diameter x 18 cm in height, 68 ml, mfd. by Pharmacia LKB Biotechnology) by passing therethrough. Subsequently, it was treated in the same manner as the one described in Example 34 to thereby give 9.1 mg of highly pure curculin.

Example 37 (treatment with hydrochloric acid)



The procedure of Example 32 was repeated except that 800 ml of the 0.0156 N sulfuric acid employed in Example 32 was replaced with 800 ml of 0.0156 N hydrochloric acid. Similar to the case of Example 32, impurities were detected in the treated solutions.

The pH values of the treated solutions 1 to 3 were respectively 3.39, 2.43 and 2.01. In this case, the precipitate (pulp fraction) suffered from little browning.

Example 38 (treatment with nitric acid)

The procedure of Example 32 was repeated except that 800 ml of the 0.0156 N sulfuric acid employed in Example 32 was replaced with 800 ml of 0.0156 N nitric acid. Similar to the case of Example 32, impurities were detected in the treated solutions.

The pH values of the treated solutions 1 to 3 were respectively 3.41, 2.45 and 2.04. In this case, the precipitate (pulp fraction) suffered from little browning.

Example 39 (treatment with phosphoric acid)

The procedure of Example 32 was repeated except that 800 ml of the 0.0156 N sulfuric acid employed in Example 32 was replaced with 800 ml of 0.0313 N phosphoric acid. Similar to the case of Example 32, impurities were detected in the treated solutions.



The pH values of the treated solutions 1 to 3 were respectively 3.50, 2.92 and 2.45. In this case, the precipitate (pulp fraction) suffered from little browning.

Example 40 (treatment with acetic acid)

The procedure of Example 32 was repeated except that 800 ml of the 0.0156 N sulfuric acid employed in Example 32 was replaced with 800 ml of 2.0 N acetic acid. Similar to the case of Example 32, impurities were detected in the treated solutions.

The pH values of the treated solutions 1 to 3 were respectively 2.90, 2.36 and 2.09. In this case, the precipitate (pulp fraction) suffered from little browning.

Example 41 (treatment with citric acid)

The procedure of Example 32 was repeated except that 800 ml of the 0.0156 N sulfuric acid employed in Example 32 was replaced with 800 ml of 165 mM citric acid. Similar to the case of Example 32, impurities were detected in the treated solutions.

The pH values of the treated solutions 1 to 3 were respectively 3.02, 2.41 and 2.14. In this case, the precipitate (pulp fraction) suffered from little browning.

Example 42 (extraction of curculin with hydrochloric



acid)

The procedure of Example 35 was repeated except that the precipitate (pulp fraction) obtained in the above Example 37 was used and that the 0.05 N sulfuric acid employed in Example 35 was replaced with 0.05 N hydrochloric acid. The following Table 8 shows the curculin concentrations and pH values of the extracts 1 to 3 thus obtained.

Example 43 (extraction of curculin with nitric acid)

The procedure of Example 35 was repeated except that the precipitate (pulp fraction) obtained in the above Example 38 was used and that the 0.05 N sulfuric acid employed in Example 35 was replaced with 0.05 N nitric acid. The following Table 8 shows the curculin concentrations and pH values of the extracts 1 to 3 thus obtained.

Example 44 (extraction of curculin with phosphoric acid)

The procedure of Example 35 was repeated except that the precipitate (pulp fraction) obtained in the above Example 39 was used and that the 0.05 N sulfuric acid employed in Example 35 was replaced with 0.75 N phosphoric acid. The following Table 8 shows the curculin concentrations and pH values of the extracts 1 to 3 thus obtained.

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[Table 8]

	Acid soln.	Extract 1 curculin concn. (pH)	Extract 2 curculin concn. (pH)	Extract 3 curculin concn. (pH)
Ex. 42	0.05 N HCl	135(1.44)	40(1.40)	9(1.39)
Ex. 43	0.05 N HNO ₃	131(1.45)	42(1.43)	11(1.41)
Ex. 44	0.75 N H ₃ PO ₄	133(1.43)	39(1.41)	8(1.40)
(unit)		(µg/ml)	(µg/ml)	(µg/ml)



CLAIMS



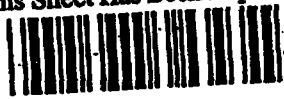
1. A process for extracting protein curculin which comprises adding an aqueous acid solution to plant bodies containing protein curculin to thereby adjust the pH of the resulting mixture to 3 or below and then extracting the protein curculin.

2. A process for extracting protein curculin as claimed in Claim 1, wherein the pH value of the mixture is 2 or below.

3. A process for extracting protein curculin as claimed in Claim 1, wherein the pH value of the mixture is from 0.5 to 1.8.

4. A process for extracting protein curculin which comprises adding an aqueous acid solution to plant bodies containing protein curculin, treating the resulting mixture (1) under such conditions as to give a

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pH value of 1.8 to 4.0 and then extracting the protein curculin from the treated mixture (2).

5. A process for extracting protein curculin as claimed in Claim 4, wherein the pH value of the mixture (1) is from 2.0 to 3.0.

6. A process for extracting protein curculin as claimed in Claim 4, wherein the pH value of the mixture (2) is adjusted to 3 or below and then the protein curculin is extracted from said mixture (2).

7. A process for extracting protein curculin as claimed in Claim 6, wherein the pH value of the mixture (2) is 2 or below.



8. A process for extracting protein curculin as claimed in Claim 6, wherein the pH value of the mixture (2) is from 0.5 to 1.8.

9. A process for extracting protein curculin as claimed in Claim 4, wherein the protein curculin is extracted from the mixture (2) with the use of an aqueous solution of a salt of a concentration of 0.01 M or above.