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[54] **PROCESS FOR ENZYMATIC DEGREASING OF BONES**
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[56] **References Cited**

UNITED STATES PATENTS

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ABSTRACT: A method is disclosed for the degreasing of animal bones from which gelatin is later to be extracted wherein the bones are subjected to the action of an aqueous alkaline solution of lipolytic enzymes (lipases) in the presence of a soluble calcium salt.

PROCESS FOR ENZYMATIC DEGREASING OF BONES

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a method for the degreasing of bones and, in particular, concerns a method whereby the bones are subjected to the action of lipolytic enzymes (lipases).

2. Description of the Prior Art

Bones from which gelatin is to be extracted must first be degreased. When bones are naturally degreased due to their exposure to the atmosphere and in particular the action of sun and rain, there is usually no need for further treatment prior to the procedure for the extraction of gelatin. Bones which come directly from the slaughter houses contain a high lipoid content which must be considerably reduced before an efficient gelatin extraction procedure can be commenced.

There are a variety of processes which are now in use for the purpose of degreasing bones. Common methods include the use of organic solvents such as alcohol, acetone, and the like, for the purpose of eliminating greases by first solubilizing them and then eliminating the grease containing solution. These solvent methods have all of the usual drawbacks related to the use of organic solvents including the danger of fire due to the flammability of many of these materials, the large quantities of solvent necessary in the procedure and the loss of bone substance [ossein] which takes place through the hydrolysis of collagen.

Other methods for degreasing bones have been attempted including some which are highly sophisticated such as the use of ultrasonics which causes the removal of lipoids which adhere to the bones. The results of this method are in some respects satisfactory but the cost of the operation is quite high as in the case where organic solvents are used.

It is desirable, therefore, that an efficient and relatively inexpensive process for degreasing bones be developed since bones are an important source of gelatin and particularly in view of the fact that meat processing operations operate on a narrow profit margin which necessitates the most efficient utilization of the animal byproducts. The method of the present invention provides such a process.

SUMMARY OF THE INVENTION

The present invention is based on the discovery that bones, particularly those having a lipoid content in the range from about 15 to 35 percent by weight, based upon bones containing 10 percent water, when treated with an aqueous solution of lipolytic enzymes (lipases), in the presence of a soluble calcium salt, effectively degrease the bones when they are contacted with such a solution at a temperature in the range from about 20° to 40° C.

The lipases useful in this invention may be of miscellaneous origin such as animal, vegetable or microbial. The animal lipases, however, are generally of pancreatic origin and are unstable and the pancreatic lipases contain proteolytic enzymes which may attach the bone's protein. Vegetable lipases present problems mainly due to the difficulty with which they are obtained.

We have found that lipases of microbiological origin are best suitable for use in the method of this invention. These lipases can be efficiently produced under normal industrial conditions and they have the ability to hydrolyze the triglycerides of long chain fatty acids which occur in bone lipoids and to transform these materials into diglycerides, monoglycerides, glycerol and fatty acids.

The quantity and type of lipase to be used will depend upon the origin of the bones, their lipoid content and the particular properties of the lipase. Similarly, the quantity of calcium salt to be used will depend upon the quantity of bones to be treated since there is in each case an optimum proportion with respect to the quantities of lipoid, lipase and soluble calcium salt. However, an excess of calcium is not harmful to the reaction and can be used in the process if necessary.

Among the lipases of microbiological origin, the fungic lipases are preferred and, in particular, the lipase of *Rhizopus arrhizus* var. *Delemar* (*Ra.D.*) because it is very stable, it has an optimum pH and an optimum temperature and it has a mode of action which makes it particularly efficient for the degreasing of bones. Other lipases which are efficiently used in this process are the lipases of *Rhizopus nigricans*, *Aspergillus niger* and *Candida cylindracea*.

It is possible and economically practical to reuse the lipasic solutions. The extent to which they can be reused will depend upon the specific activity of the enzyme (measured by titration as μ/g.; method described in Bulletin de la Societe de Chimie Biologique, T. 48 N. 6, 1966, pp. 747–770).

Once the residual activity has been determined, a decision can be made as to whether or not further use can be made of the solution. In general, an amount of enzyme sufficient to hydrolyze the lipoids in the bones is determined and the lipasic solution reused as long as its activity remains sufficiently high to effectively hydrolyze the lipoids.

After the treatment, the enzymatic solution is separated from the bones which are washed with water, preferably hot, to eliminate the sticking products of the reaction which would tend to inhibit the action of the lipase during subsequent treatments. The bones which have been subjected to the degreasing treatment described hereinabove are ready for further treatment, particularly the extraction of gelatin.

DESCRIPTION OF THE PREFERRED PRACTICE OF THE INVENTION

To produce high-quality gelatin, certain factors must be taken into consideration. The bones to be degreased should be treated at a pH in the range from between about 5 to about 8, the temperature should be maintained in the range from between about 20° to about 40° C. and the treatment, which may vary in this process anywhere from 3 to 20 hours, will depend upon the lipoid content in the bones and the activity of the particular lipase used in the process. For example, when lipase *R.a.D.* is used, the treatment is continued for a period of from between 6 to 12 hours for an enzyme titrating about 10,000 μ/g. specific activity. For a definition of this term see the reference above cited.

It is desirable in the process to use bones which are subdivided into pieces which increases the surface of bone in contact with the lipasic solution. Bone pieces which are able to pass through a sieve having square meshes of 16 mm. or 19 mm. on a side are preferred.

It is advisable that prior to the enzyme treatment, the previously crushed bones (called "green bones"), be subjected to a pretreatment with hot water for a period of time ranging from about 15 to 90 minutes. This results in the washing out of part of the lipoids which adhere to the bones while some residual matter remains. The pretreatment may be repeated if desired.

Each kilogram of bones to be degreased is treated with an aqueous solution of from 0.2 to 10 g. of lipase, the volume of the lipase generally ranging from between 0.5 to 3.0 liters per kilogram of bones.

A quantity of soluble calcium salt, preferably calcium chloride, is added to the lipase solution in an amount ranging from 0.2 to 10 g. of calcium salt per kilogram bones. The calcium aids in the elimination of fatty acids resulting from the action of the lipase by combining with these acids and eliminating them from the reaction medium which thus enhances the action of the enzyme; it forms a part of the enzyme molecule and acts as a specific promoter of the lipase; and it protects the enzyme and improves the effectiveness and economy of the process.

During the treatment, the bones are gently stirred, either continuously or intermittently, in the lipase solution, thus effecting maximum contact between the lipase solution and the bones. After sufficient contact has been made and the treatment completed, the enzymatic solution is separated from the bones which are then washed with hot water to eliminate the

reaction products which otherwise would tend to inhibit the action of the lipase in further treatment. The washing, which may be carried out at a pH of from between 7 to 8.5, and preferably between pH 8 and pH 8.5, may be repeated as many times as necessary.

Further to illustrate this invention, specific examples are described hereinbelow:

EXAMPLE I

Slaughterhouse bones having a lipoid content of 23.2 percent (based on bones containing 10 percent water) were crushed and passed through a 19 mm. mesh sieve. The bones were washed in boiling water for 30 minutes, after which the liquid containing lipoids and meat parts was eliminated and the operation repeated.

After elimination of the adherent meat remains and then draining, the partly degreased bones, which were thus obtained, were ready for lipasic treatment.

One kilogram of the bones was introduced into 1 liter of an aqueous solution of lipase *R.a.D.* containing 0.5 g./l. of lipase (activity = 9,000 μ/g.) together with 3 g. of $CaCl_2$. The treatment was continued for 6 hours at 37° C. while the solution was continuously stirred. The pH was continuously maintained at 7.2±0.2 by adding an aqueous solution of 0.5 M NaOH.

When the first lipasic treatment was ended, the solution was drawn off and the bones were stirred in water at 75° C. for 15 minutes, after which another lipasic treatment as above was commenced, using 0.5 g. of lipase, (activity = 9,000 μ/g.) together with 9 g. of $CaCl_2$ per kilogram of bones. The third and last treatment with lipase was followed by a last water washing at 75° C.

Results:

Percentage of lipoids based on bones containing 10 percent water:

initially	= 23.2%
after the pretreatment	= 14.8%
after the 1st lipasic treatment	= 8.5%
after the 2nd lipasic treatment	= 4.6%
after the 3rd lipasic treatment	= 1.5%
Percentage of lipoids removed:	about 94.5%

EXAMPLE 2

Slaughterhouse bones having a lipoid content of 19 percent (based on bones containing 10 percent water) were treated. The bones were constituted as follows: half of the pieces being about 16 mm. in size and half being about 19 mm. They were washed in boiling water for 40 minutes, after which the solution containing lipoids and meat remains was eliminated.

One hundred kilograms of the bones which had been subjected to this pretreatment were introduced into 100 liters of an aqueous solution of lipase *R.a.D.* containing 0.8 g./l. of lipase, (activity = 7,500 μ/g., i.e., 6,000 μ/kg. of bones) and also 6 g./l. of $Cacl_2$. This treatment was continued for 12 hours at 37° C., and the pH maintained at 7.2±0.2 by the addition of a 0.5 M solution of NaOH.

After the first lipasic treatment, the solution was drawn off and the bones stirred with water at 70° C. for 20 minutes, the water having been brought to and then maintained at pH 8.5 by the addition of a dilute aqueous solution of NaOH.

After eliminating the rinsing solution, a second treatment was commenced with a new aqueous solution of lipase *R.a.D.* containing 2.5 g. of lipase (activity = 7,500 μ/g. per kilogram of bones, i.e., about 18,000 μ/kg. of bones) and 9 g./l. of $CaCl_2$, the quantities of solutions being the same comparatively to the weight of bones as in the first treatment.

The second treatment was continued for 12 hours at 37° C., the pH maintained, as in the first treatment, at 7.2±0.2 by a solution of 0.5 M NaOH.

After the second treatment, the bones were rinsed as before.

Results:

Percentage of lipoids based on bones containing 10 percent water:

initially	= 19.0%
after the pretreatment	= 11.0%
after the 1st treatment (lipasic)	= 5.8%
after the 2nd lipasic treatment	= 1.5%
Lipoids removed:	about 92%

EXAMPLE 3

Crushed slaughterhouse bones, about 19 mm. in size and containing 35.3 percent of lipoids based on bones containing 10 percent water, were subjected, in a pretreatment, to the washing action of boiling water for 30 minutes after which the solution containing lipoids and meat remains was eliminated. A second pretreatment was then commenced by subjecting the bones to the action of boiling water for 60 minutes.

Two thousand kilograms of the pretreated bones were placed into 1,700 liters of a solution containing 3.5 g./l. of lipase *R.a.D.*, (activity = 12,000 μ/g., i.e., about 31,000 μ/kg. of bones) and 10.6 g. of $CaCl_2$ per liter of solution.

The treatment was continued for 8 hours at 37° C., the pH being maintained at 7.2±0.2 by the addition of 0.5 M KOH.

After the first treatment, the lipasic solution was drawn off and the bones stirred for 30 minutes with water brought to 80° C., the pH of which was maintained at 8.4 by the addition of a dilute solution of KOH. The alkaline rinsing solution was eliminated and a second rinsing commenced with water at 75° C., with vigorous stirring.

After elimination of the second rinsing solution, a second lipasic treatment was commenced with 2,000 liters of a lipasic solution containing 1 g./l. of lipase of *R.a.D.* (activity = 12,000 μ/g., i.e., 12,000 units per kilogram of bones) and 6 g./l. of $CaCl_2$; the treatment was continued for 10 hours at 37° C., while maintaining the pH at 7.2±0.2 by the addition of an aqueous solution of 0.5 M KOH.

After the second lipasic treatment, the bones were rinsed under the same conditions as previously described. A third treatment was then commenced with 2,000 liters of a solution of lipase of *R.a.D.* (activity = 12,000 μ/g., i.e., 3,400μ per kilogram of bones) and 6 g./l. of $CaCl_2$, followed by the rinsing as described hereabove.

Results:

Percentage of lipoids based on bones containing 10 percent water:

initially	= 35.3%
after the pretreatment (double)	= 24.1%
after the first lipasic treatment	= 11.3%
after the 2nd lipasic treatment	= 5.4%
after the 3rd lipasic treatment	= 1.1%
Lipoids removed:	about 97%

EXAMPLE 4

Slaughterhouse bones comprising a fairly equal per part mixture of 16 mm. and 19 mm. pieces, containing 29.3 percent of lipoids (based on the bones containing 10 percent water) were subjected to the washing action of boiling water for 30 minutes, after which this solution containing lipoids and meat remains was eliminated.

To 1,000 kilograms of these pretreated bones was added 1,500 liters of a 1 g./l. aqueous solution of lipase of *Candida cylindracea*, (activity = 10.500 μ/g., i.e., 15,750μ per kilo of bones), 4 g./l. of $CaCl_2$ and 2 g./l. of $MgCl_2$; this treatment was continued for 10 hours at 34° C., the pH being maintained at 7±0.2 by the addition of an aqueous solution of 0.5 M NaOH. This pH was an average between the optimum pH of the enzyme which is 6.7 and the pK value (dissociation constant) of the fatty acids.

After the treatment was completed, the solution was drawn off and the bones were rinsed by stirring in water at 75° C. for 40 minutes, the pH being brought to and maintained at 8.5 by the addition of an aqueous solution of dilute NaOH. The

rinsing solution was eliminated, and a second rinsing commenced with water brought to 75° C.

A second treatment was then commenced identical to the first, and continuing for 15 hours, after which the bones were rinsed under the same conditions as above.

Results:

Percentage of lipoids based on bones containing 10 percent water:

initially	= 29.3%
after the pretreatment	= 20.1%
after the 1st lipasic treatment	= 8.7%
after the 2nd lipasic treatment	= 1.8%
Lipoids removed:	about 94%

EXAMPLE 5

Crushed slaughterhouse bones of about 16 mm. size and having a percentage of lipoids amounting to 25 percent based on bones containing 10 percent water were subjected for 60 minutes to the action of boiling water. After elimination of the water, the operation was repeated a second time.

One thousand kilograms of the treated bones were placed into 10,000 liters of an aqueous solution of lipase of *Rhizopus nigricans* containing 1 g./l. of enzyme (activity = 5,200 μ/g., i.e., 5,200μ per kilogram of bones) and 3 g./l. of $CaCl_2$.

The treatment was continued for 8 hours at 28° C., the pH being maintained at 6.9±0.2, this pH being an average between the optimum pH of the enzyme and the pK of the fatty acids.

During this treatment, the stirring was slow and discontinuous.

At the end of the treatment, the solution was eliminated and the bones rinsed with 10,000 liters of water brought to 70° C. for 15 minutes, the pH of which was maintained at 8.5 by the addition of a dilute solution of NaOH.

A second treatment was commenced with 10,000 liters of a solution of lipase containing 1 g./l. of lipase of *Rhizopus nigricans*, (activity = 5,200 μ/g., i.e., 10,400μ per kilogram of bones) and 3 g./l. of $CaCl_2$.

The treatment was continued for 10 hours at 28° C. under the same conditions as in the first treatment, followed by a rinsing, as previously done.

Finally, a last treatment was commenced, under the same conditions as above, but using a solution containing 3 g./l. of enzyme. Another rinsing was then commenced with water brought to pH 8.5, and at a temperature of 80° C.

Results:

Percentage of lipoids based on bones containing 10 percent water:

initially	= 25.0%
after the pretreatment	= 18.5%
after the 1st lipasic treatment	= 9.8%
after the 2nd lipasic treatment	= 5.1%
after the 3rd lipasic treatment	= 1.4%
Lipoids removed:	about 94.4%

EXAMPLE 6

Crushed slaughterhouse bones of 19 mm. size and having a lipoid content of 23.4 percent based on bones containing 10 percent water were subjected to the action of boiling water for 45 minutes. After treatment the liquid was eliminated and an identical treatment commenced.

Five hundred kilograms of the treated bones were placed into 500 liters of an aqueous solution of lipase of *Aspergillus niger* containing 10 g./l. of enzyme (activity = 2,400 μ/g., i.e., 24,000μ per kilogram of bones) at 30° C., the pH being maintained at 7.2±0.2 by the addition of an aqueous solution of 0.5 M NaOH; the treatment included a discontinuous stirring for 15 hours.

At the end of the treatment, the solution of lipase was drawn off after which 50 liters of water brought to 80° C. was added, the pH of which was maintained at 8.5 by the addition of dilute NaOH, accompanied by stirring for 15 minutes; the solution was then eliminated, and a second rinsing under the same conditions commenced.

A second lipasic treatment was commenced with 759 liters of an aqueous solution containing 2.1 g./l. of lipase (i.e., 7,700μ per kilogram of bones), and 2 g./l. of $CaCl_2$.

The treatment was continued with discontinuous stirring for 15 hours at 30° C., the pH being maintained at 7.2±0.2 by the addition of a solution of a 0.5 M NaOH. This treatment was followed by a rinsing.

Results:

Percentage of lipoids based on bones containing 10 percent water:

initially	= 23.4%
after the pretreatment	= 16.8%
after the 1st lipasic treatment	= 8.4%
after the 2nd lipasic treatment	= 1.3%
Lipoids removed:	about 94.5%

We claim:

1. A method for the enzymatic degreasing of bones which comprises subjecting said bones to the action of a lipolytic enzyme solution in the presence of a soluble calcium salt.

2. A method according to claim 1 wherein said lipolytic enzyme solution is at a pH in the range from about 5 to about 8 and at a temperature in the range from about 20° to about 45° C.

3. A method according to claim 2 wherein said lipolytic enzyme is of microbiological origin.

4. A method according to claim 2 wherein said lipolytic enzyme is a member of the group consisting of the lipases of *Rhizopus arrhizus Delemar*, *Rhizopus nigricans*, *Aspergillus niger*, *Candida cylindracea*, *Mucor mucedo*, *Rhizpus oryzae*, *Candida lipolytica* and *Aspergillus lucheunsis*.

5. A method according to claim 2 wherein said soluble calcium salt is calcium chloride present at a concentration of from 0.2 g. to 10 g. per kilogram of bones.

6. A method according to claim 2 wherein prior to degreasing, the bones are washed in boiling water at an alkaline pH.

7. A method according to claim 2 wherein the bones are subjected to repeated contact with the lipolytic enzyme solution.

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