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[54] **SOLUBILIZATION OF INSOLUBLE COLLAGEN**
5 Claims, No Drawings

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[50] **Field of Search** 195/6

[56] **References Cited**

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ABSTRACT: A method of solubilizing insoluble collagen which comprises treating said insoluble collagen at a temperature of 0°~37° C., at a pH of 1.5 to 3.0 with the collagen solubilizing proteolytic enzyme produced from specific micro-organisms having an optimum activity for solubilizing milk casein at a pH of 2.2 to 2.4.

SOLUBILIZATION OF INSOLUBLE COLLAGEN

CROSS REFERENCES TO RELATED APPLICATION

This application is a continuation-in-part of copending application Ser. No. 403,357, filed on Oct. 12, 1964 now abandoned, Ser. No. 561,730 filed on June 30, 1966 now abandoned and Ser. No. 624,229 Mar. 20, 1967 now abandoned, entitled to the priority date of Japanese Pat. application Nos. 54240/63 and 55237/64 respectively filed on Oct. 11, 1963 and Sept. 30, 1964.

This invention relates to a method of solubilizing so-called insoluble collagen with a proteolytic enzyme having activity in the acidic range. More particularly, this invention relates to a method of solubilizing insoluble collagen by effecting the enzyme treatment and the acid extraction of the collagen simultaneously. When insoluble collagen is treated with a proteolytic enzyme at a temperature less than that at which the collagen fibers shrink and thereafter extracted with dilute acid, the collagen is dispersed therein, and thus a solution containing undenaturated collagen molecules having a rigid rod helical structure can be obtained. When a proteolytic enzyme having activity in the neutral or alkaline range is used in the solubilization of insoluble collagen, the insoluble collagen is first treated with the enzyme and thereafter extracted with dilute acid to obtain a collagen solution. However, when a proteolytic enzyme having activity in the acidic range is used in the solubilization of insoluble collagen, then, because the pH range in which the enzyme acts upon the insoluble collagen and the pH range in which the insoluble collagen is dissolved by the action of the enzyme correspond, both the enzyme treatment and the acid extraction can be carried out simultaneously. Thus, this invention is very advantageous for the solubilization of insoluble collagen on an industrial scale.

Pepsin and the proteolytic enzyme produced from *Paecilomyces varioti* are well known in the art. These enzymes have an optimum pH in the acidic range. It has now been found that certain species of molds belonging to the genus Penicillium produce proteolytic enzymes having a very high ability to solubilize collagen. These species are *Penicillium spinulosum*, *Penicillium frequentans* and *Penicillium citreo-viride*.

Accordingly, the present invention provides a method of solubilizing insoluble Collagan in undenatured form which

comprises mixing a proteolytic enzyme produced from the moulds of *Penicillium spinulosum*, *Penicillium frequentans* and *Penicillium citreo*-viride with the insoluble collagen in an acidic medium. These proteolytic enzymes also have an optimum pH in the acidic range.

Although pepsin and the proteolytic enzyme produced from *Paecilomyces varioti* have the ability to solubilize collagen, these enzymes dissolve only about 15 to 20 percent by weight of collagen, whereas the enzymes employed in the method of the present invention dissolve at least 65 percent by weight, and in certain cases more than 90 percent by weight, of collagen in an acidic medium.

The proteolytic enzymes employed in the method of the present invention are generally used in an amount of about 0.01 to 1 percent by weight of the insoluble collagen at a temperature of from 0° to 37° C., preferably at about 25° C., and in the pH range of from about 1.5 to 3.0.

The ability of the proteolytic enzymes employed in the method of the present invention to solubilize insoluble collagen are shown in the following table. The ability of pepsin and the proteolytic enzyme produced from *Paecilomyces varioti* to solubilize insoluble collagen are also shown for comparison purposes only.

TABLE

Strains	Enzyme	Ability of enzyme to solubilize collagen, percent [1]
Penicillium spinulosum T-4	Filtrate of the fermentation broth.	93
Penicillium spinulosum T-4	The first fraction of above filtrate.	96
Penicillium frequentans 47-4	Filtrate of the fermentation broth.	96
Penicillium citreo-viride 36-7	do	95
Paecilomyces varioti	do	15
	Pepsin [2]	22

[1] The activity of the enzyme is measured by the percentage of collagen dissolved after digestion for 24 hours under constant agitation in an aqueous solution of 0.2M acetic acid at 25° C. The amount of enzyme employed was sufficient to digest casein at a rate equivalent to 1% pepsin on casein.

[2] Purified pepsin was used.

The strains *Penicillium spinulosum* T-4, *Penicillium frequentans* 47-4 and *Penicillium citreo-viride* 36-7 are deposited as A.T.C.C. (American Type Culture Collection) Nos. 16348,

MYCOLOGICAL CHARACTERISTICS OF EACH STRAIN

	Strain No.			
	T4	7-5	47-4	36-7
ATCC No.	16,348	16,349	16,450	16,451.
(1) Growth rate of giant colony (diameter of colonies 25° (10 days)				
Czapek agar	33 mm	52 mm	50 mm	47 mm.
Czapek steep agar	51 mm	76 mm	58 mm	68 mm.
(2) Morphological characteristics of giant colony				
Texture	Velvety	Velvety	Velvety	Velvety.
Character of surface	Radical, furrowed deeply, smooth, raised in central area.	Smooth, slightly raised in central area, conidial, chain twisting.	Smooth, slightly raised in central area.	Radical, furrowed deeply.
Character of margin	1-1.3 mm., white	2.0 mm., white	0.5-3.5 mm., white, greenish white.	0.5-1.0 mm., white, bluish white.
Color change	Greenish blue green→ greenish blue green or brownish blue green.	Greenish blue green→ brownisn gray green.	Greenish blue green→ gray green yellow or brownish green.	Greenish blue green→ gray green yellow or grayish brown.
Conidia formation	Abundantly	Very abundantly	Very abundantly	Poor.
Exudate	Limited (reddish purple)	Lacking	Lacking	Lacking.
Colony reverse	Pale orange yellow→gray yellow.	Pale orange yellow→orange yellow.	Yellowish green yellow→ pale pinkish yellow.	Pale yellow green→gray green or gray yellow.
Pigmentation of substratum	Reddish purple	Colorless	Colorless	Colorless.
(3) Microscopic characters				
Penicillus	Mono-verticillata strictly stand from substratum.	Mono-verticillata	Mono-verticillata	Mono-verticillata.
Conidiophore:				
Length	50-200μ	40-80μ	30-100μ	16-60μ.
Diameter	2.6μ	2.7μ	2.4μ	2.8μ.
Surface	Smooth	Smooth	Rough	Smooth.
Sterigmata: Usual Number	6-10	8-12	6-10	4-6.
Arrangement	Compact	Compact	Compact	Compact.
Length	9.5-13μ	8-11μ	8-10μ	8-10μ.
Diameter	2.5μ	2.2μ	2.4μ	2.7μ.
Surface	Smooth	Smooth	Slightly rough	Smooth.
Conidia:				
Form	Globose	Globose	Globose	Globose.
Size	2.2-4.8μ	1.6-3.2μ	1.6-4.8μ	1.9-3.7μ.
Marking	Smooth or granular	Smooth	Smooth	Smooth.
Others—Perithecia and sclerotia, not observed				
Species of penicillium identified	Spinulosum	Frequentans	Frequentans	Citreoviride.

16349/50 and 16351, respectively, and were released for public distribution on Aug. 18, 1969.

In accordance with the present invention, the solubilization of insoluble collagen can be carried out with an enzyme produced from any of the above listed strains of Penicillium, and an inorganic acid, e.g., hydrochloric, sulfuric or phosphoric acid, or an organic acid, e.g., citric or acetic acid. However, of these acids, the use of hydrochloric acid is advantageous from an industrial standpoint.

The *Penicillium spinulosum* strain mentioned above is isolated from vegetable tanning liquor. When cultivated in a medium composed mainly of wheat bran, it produces little amylase, the enzymes being largely acid protease. The acid protease of this strain or of other superior strains belonging to Penicillia, as will be shown in the examples to be cited hereinafter, is readily produced by the solid cultivation method. The aerobic submerged cultivation method may also be used. However, of these two methods, the latter is advantageous since the time required for accumulation of a large amount of enzyme is remarkably shortened. Moreover, the amount of enzyme accumulated is extremely large, and the extraction-purification procedure of enzyme is simplified. It is recognized, therefore, that these strains are suitable for the aerobic submerged culture and are desirable strains for the industrial production of the proteolytic enzymes mentioned in table 1.

The medium to be used for cultivation must contain the usual carbon source, nitrogen source, inorganic substances and other substances. The use of an inorganic nitrogenous substance alone as the nitrogen source results in a reduced enzyme production. Hence, the use of an organic nitrogen source is preferable. Wheat bran is particularly effective to increase enzyme accumulation.

The amount of enzyme accumulated was examined by using a wheat bran containing medium as the base and adding thereto phosphate and inorganic substances in various combinations. The accumulation of enzyme was increased by the addition of monopotassium phosphate. Further, when calcium or magnesium salt was added in a small amount, the conditions of accumulation of enzyme were stabilized. Other inorganic salts produced no significant effect. When starch was added, the accumulation of enzyme tended to increase slightly.

The cultivation was carried out at a temperature of 28° C. If the temperature is raised several degrees higher than 28° C., the enzyme accumulation increased within a short cultivation period. Also, if the initial pH of the culture medium is less than 3.5 or more than 6.0, the growth of this strain is poor and, accordingly, the amount of enzyme accumulated is small. The suitable initial pH is 4.0–5.0, in which case the final pH is usually 4.5–5.5.

Under suitable cultivation conditions as described above, following an induction period of about 10–16 hours following the inoculation of the seed culture, the growth thereof proceeds into the logarithmic phase. After about 30 hours corresponding to this phase, the amount of enzyme accumulated begins to increase, reaching the maximum activity within about 50–56 hours. After 60 hours, the enzyme activity tends to decrease gradually. During that time, the pH of culture liquid is in the range of 5.0 to 5.5, showing no great change.

The use of a fermentation broth having the maximum enzyme activity is preferable since the impurity content therein is less.

When a crude enzyme preparation obtained by the fractionation of ammonium sulfate from the culture liquid of this strain cultivated in a medium composed mainly of wheat bran was developed chromatographically with an acetate buffer solution of pH 3.5 on a column of a weakly acidic cation exchange resin bufferized with the same buffer solution.

The mycological characters of the strains of Penicillium shown in the above table, and the process for obtaining enzymes therefrom are disclosed in our Application Ser. No. 714,355, filed on Mar. 19, 1968, which is a continuation-in-part of copending application Ser. No. 469,865 filed on July 6, 1965 both now abandoned. The above-mentioned micro-organisms are cultivated by the solid cultivation method or the aeration-stirring submerged cultivation method using wheat bran. The pH value, which at the commencement of cultivation ranges from 3.5 to 6.0, preferably 4.0 to 5.0, changes to within the range of 5.0 to 5.5 at the end of the cultivation process. The crude enzyme preparation obtained by fractionation with ammonium sulfate from the culture liquid fermentation broth is passed down a column of a weakly acidic cation exchange resin. Preferred resins are:

(Amberlite IRP–64, made by Rohm & Haas Co., U.S.A.; Duolite CS–101, made by Chemical Process Co., U.S.A.; Carboxymethyl-cellulose, made by Brown Co., U.S.A.; Selva-Entwicklungslabor., Germany; or Whatman Co., England; or CM Sephadex, AB. Pharmacia, Sweden).

In accordance with the ordinary method, a first acid protease fraction was eluted and, thereafter, when the pH of the eluted solution was raised, a second fraction was eluted at around pH 5.0. The first fraction was obtained in a larger amount than the second fraction in a ratio of more than 10:1 at all times.

Upon examination of these two fractions, the peak of each, when examined by ultracentrifugation, was considered almost homogeneous. When these fractions were further purified and crystallized by procedure of example 3, sedimentation constants (S_{20w}) of the first fraction and the second fraction were calculated from Svedberg's equation as 3.1S and 3.4S respectively. The first fraction was found to have an optimum pH of about 2.2 to 2.4 for digesting milk casein, and the second fraction had an optimum pH of about 3.2. Also, it was observed that the ability to solubilize the collagen of native steer hide is much stronger in the first fraction than in the second fraction.

The use of the enzymes of *P. spinulosum* to degrade native proteins including albuminoid other than the collagen was examined using hemoglobin, globin, albumin, globulin, gelatin, keratin, elastin, or fibrin as the insoluble protein. It was found that the two fractions do not differ much as compared with the acid protease hitherto known. But, upon examination of the milk-clotting activity of both fractions, it was observed that the second fraction shows an extremely low milk-clotting activity, whereas the first fraction shows a strong milk-clotting activity, it having an activity superior to that of the acid protease produced from *Aspergillus saitoi*. The optimum pH, stable pH range, thermal resistance and optimum temperature for the enzyme to digest milk casein are shown below

	1st Fraction	2nd Fraction
Optimum pH	About 2.2–2.4	About 3.2
Stable pH Range	2.0–5.5	3.0–6.0
Thermal Resistance (Temperature at which activity is lost)	55° C., 10 min.	55° C., 10 min.
Optimum Temperature	about 45° C.	about 45° C.

Also, the effects of an inhibitor and metallic salt upon the ability of the enzyme of *P. spinulosum* to digest milk casein were examined. It was found that this ability was inhibited by sodium lauryl sulfonate and iodine in the same way as in the case of the acid protease produced from *Aspergillus saitoi*. The effects of EDTA (ethylene diamine tetraacetic acid) and other inhibitors were not observed, and, also, various kinds of metal ions had no effect. From these points, no difference between the two fractions was observed.

Summed up, of the two fractions, the second fraction is similar in many points such as the optimum pH, etc. to the acid protease usually produced from the known fungi, whereas the first fraction differs, particularly in the optimum pH thereof and in the ability to solubilize collagen, from the acid protease produced from the known Aspergillus series or Penicillia.

The four strong acid protease-producing strains belonging to Penicillia in this invention each produce a first fraction which is effective for the solubilization of collagen in a larger amount than the second fraction by the solid cultivation or aerobic submerged cultivation method. The first fraction is characterized by an optimum pH for digesting casein of 2.2 to 2.4.

Aspergillus saitoi was made available for public distribution under ATCC No. 14 332 and is described in U.S. Pat. Nos. 3,149,051 and 2,848,371.

The enzyme absorbed on the resin is then eluted with a sodium acetate buffer solution having a pH of 3.5, and is extracted from the eluate by, for example, the freeze drying method.

EXAMPLE 1

60 g. of wheat bran was placed in each of 20 Fernbach flasks of 1 liter capacity, plugged with cotton and thereafter sterilized at 180° C. in advance. Thereafter, the bran in each flask was sprinkled with 60 ml. of water and subsequently sterilized twice for 1 hour under a pressure of 1 kg./cm².

At the same time, the preserved culture of the above mentioned *Penicillium spinulosum* T–4 strain was inoculated into a culture medium prepared in the same way by using 10 g. of wheat bran and cultivated for 5 days to form spores, thereby preparing a seed culture. 50 ml. of sterilized water was added to each culture to prepare a spore suspension aseptically.

5 ml. of the spore suspension was inoculated into each of the first-mentioned wheat bran culture media and permitted to grow on the solid cultivation medium for 5 days at 28° C. to grow the spores fully. 4.8 liters of aqueous acid solution adjusted to ph 3.5 with hydrochloric acid or acetic acid was then added to the solid-cultivated substance and allowed to stand overnight in a refrigerator kept at 3°–5° C. to extract acid protease. Thereafter, it was filtered by using gauze or cotton cloth to separate the culture residue which was washed further with 1.2 liters of aqueous acid solution of pH 3.5, the washings being added to the filtrate.

The activity of the resulting liquid was 460 tyrosine γ/ml., in terms of the degree of casein digestion at pH 2.7, as measured by the Folin's reagent. Subsequently, calcium acetate was added to this liquid so as to make the final concentration 1/10 molar, and the mixture was held for from 30 minutes to 1 hour. It was then filtered to remove the precipitate which formed together with the calcium acetate. Thereafter, ammonium sulfate powder was dissolved in the supernatant liquid in an amount sufficient to provide a solution 40 percent saturated while the latter was cooled and stirred, the resulting mixture being left alone overnight in a refrigerator. The precipitate here formed was removed by using centrifugation. Thereafter, further ammonium sulfate was added to said supernatant liquid while adequately stirring and cooling until the final concentration reached 85 percent saturation. The mixture was held 48 hours in a refrigerator so as to fully salt out the active enzyme fraction having an ammonium-sulfate saturation of 0.4–0.85. After salting out, the precipitated enzyme fraction was collected, then dissolved in a small amount of water (pH 3.5), thereafter charged chromatographically on a column of Sephadex G–25 and eluted with distilled water. The enzyme-protein fraction that was eluted first was collected by a fraction collector and dried by the freeze-drying method to obtain a crude enzyme preparation. The yield of the active enzyme, in terms of the degree of casein digestion at pH 2.7, was about 40 percent of the amount accumulated after cultivation. This preparation had almost the same collagen-solubilizing activity as that stated hereinbefore.

EXAMPLE 2

750 g. of wheat bran was suspended in 14 liters of water, then sterilized twice intermittently for 1 hour under a pressure of 1 kg./cm.², thereafter transfused into a presterilized small-scale fermentation tank, and, with the pH being adjusted to 4.0, further sterilized by steaming for 1 hour under a pressure of 2 kg./cm.² and thereafter cooled to 28° C.

At the same time, in the same way as in example 1, 25 g. of wheat bran was taken in each of six Fernbach flasks of 1 liter capacity, followed by the addition of 25 ml. of water, then sterilized, and, thereafter, the preserved culture of *Penicillium spinulosum* T–4 strain was inoculated thereinto and cultivated for 6 days at 28° C. to form spores sufficiently, thereby preparing a seed culture. From this seed culture, the spores were taken out aseptically, then suspended in 1 liter of sterilized water and thereafter inoculated into the above-mentioned fermentation tank.

After inoculation, the organism was allowed to grow under submerged cultivation conditions for 50 hours at an agitation rate of 400 r.p.m. and a rate of aeration of 10–15 liters/min., at 28° C. The protease activity of the culture liquid after completion of cultivation was about 400 tyrosine γ/ml. in terms of the degree of casein digestion at pH 2.7, it being observed that the enzyme had accumulated in an amount about four times per 1 g. of wheat bran as compared with the case of the solid cultivation in example 1.

Subsequently, the culture liquid was filtered through filter cloth. The pH of the filtrate was adjusted to 3.5 with acetic acid or hydrochloric acid, followed by the addition of calcium acetate so as to make the final concentration 1/10 molar. The precipitate here formed was filtered by suction. Then, ammonium sulfate powder was added to the filtrate to a concentration of 40 percent saturation, and the mixture was salted out overnight at 3°–5° C. The precipitate here formed was separated by filtration, and immediately thereafter, more ammonium sulfate powder was added to the supernatant liquid obtained and, while stirring and cooling, dissolved therein until 90 percent saturation was reached. Thereafter, the liquid was held for 2 days at 3°–5° C. and salted out fully. After salting out, the resulting precipitate fraction of 0.4–0.9 saturation, in which the greater part of the active enzyme had gathered, was collected by successive centrifugation and dissolved in a small amount of aqueous hydrochloric acid solution (pH 3.0). The calcium acetate fraction which was then formed simultaneously was washed and concurrently separated by centrifugation, and the enzyme fraction of brown color was collected.

The crude enzyme liquid was charged and eluted chromatographically on a column (diameter 8 cm.×length 70 cm.) of Sephadex G–25 at a flow rate of 400 ml./hr. and thereby separated from the ammonium sulfate fraction to obtain a desalted enzyme fraction. This fraction was then freeze-dried to obtain crude enzyme powder. This powder had the same collagen-solubilizing ability as that shown hereinbefore, but the yield of the active enzyme from the culture filtrate was about 60 percent in terms of the degree of casein digestion at pH 2.7.

EXAMPLE 3

1.5 kg. of wheat bran was suspended in 8 liters of water, then sterilized in the same way as in example 2, thereafter transfused into a presterilized fermentation tank, followed by the addition of a solution containing 15 g. monopotassium phosphate (KH_2PO_4) and 1.5 g. calcium chloride, thereby adjusting the pH to 4.0. The liquid, having a total volume of 28 liters, was then sterilized under pressure in the same way as in example 2. The liquid was then cooled, and, thereafter, 2 liters of the spore suspension from the seed culture of *Penicillium spinulosum* T–4 strain prepared in the same ratio as in example 2 was inoculated and allowed to grow under submerged cultivation conditions for 50 hours at an agitation rate of 400 r.p.m. and a rate of aeration of 20–30 liters/min. at 28° C. The activity of the protease after completion of cultivation was 800–1,000 tyrosine γ/ml. in terms of the degree of casein digestion at pH 2.7, and the amount of acid protease accumulated was large as compared with the case of a medium composed of wheat bran alone.

The amount of enzyme accumulated can be increased to 1,500–1,900 tyrosine γ/ml. through the selection of the best quality seed strains from the *Penicillium spinulosum* cultures

and by optimizing the seed culture inoculation conditions.

After completion of cultivation, the culture liquid was put in a filter bag and compressed by a small-scale squeezer. The expressed liquid was filtered, the pH of the filtrate was then adjusted to 3.5, and calcium acetate was added to a concentration of 1/40 molar. The mixture stood for about 1 hour at a low temperature. The precipitate here formed was filtered in a small-scale filter press, and ammonium sulfate powder was added to the filtrate while stirring at a low temperature so as to make the saturation 0.4, and the mixture was held overnight at 3°–5° C. After removal of the resulting precipitate by a small-scale filter press, more ammonium sulfate powder was added to and dissolved in the supernatant liquid under full stirring at a low temperature until the 90 percent saturation was reached, and the mixture was held for 2 days at 3°–5° C. to fully salt out the enzyme fraction of 40–90 percent saturation. The precipitate here formed was collected by successive centrifugation and dissolved with an appropriate amount of aqueous acid solution (pH 3.5).

This crude enzyme solution was then charged and eluted chromatographically on a column (diameter 8 cm.×length 70 cm.) of Sephadex G–25 (made by AB Pharmacia, Sweden), thereby separated from the ammonium sulfate fraction, and desalted. (The use of Sephadex G–75 makes it possible to separate the crude enzyme solution from the colored substance and the impure protein fraction to some extent.) The acid protease fraction here obtained was collected and freeze-dried to obtain powder of a crude enzyme preparation. The yield of the active enzyme was 65 percent in terms of the degree of casein digestion at pH 2.7, said enzyme showing a high solubilizing ability as is mentioned hereinabove for the collagen of adult steer hide.

Subsequently, 5 g. of the crude enzyme preparation here obtained was charged on a column (diameter 3 cm.; final amount of resin 500 ml.) of Amberlite IRP–64 bufferized fully in advance with a 1/50 molar acetate buffer solution of pH 3.5, followed by the pouring thereinto of 1.5 liters of the same buffer solution at a flow rate of 30 ml./h.4., and developed chromatographically. The eluted solution was fractionally collected by 10–15 ml. with a fraction collector. After the first acid protease fraction was eluted, the buffer solution for elution was converted to a 1/10 mole acetate buffer solution of pH 5.2. After approximately 2 liters of this solution were passed through the column, the pH of the eluted solution became about 5.0 and a second acid protease fraction was collected. The first fraction, as previously mentioned, had an optimum pH of about 2.2–2.4, most suitable for digesting casein, and, also, had a strong ability to solubilize collagen. The second fraction had the optimum pH of about 3.2 and was inferior in the collagen-solubilizing ability. The yield of the first and second fractions was 82 percent in the former and 7.2 percent in the latter in terms of the degree of casein digestion. Protein fractions worthy of note other than said two fractions were not observed.

The two protease fractions were successively dialyzed with a 1/100 mole acetate buffer solution of pH 4.0 and through exchange of the buffer solutions, then adsorbed on a column of DEAE cellulose bufferized in advance with the same buffer solution, and thereafter, chromatographed by the method of gradient elution on ionic strength with the same buffer solution as the initial solution (1 liter) and the same solution containing 0.5 mole sodium chloride as the final solution (1 liter). (Column size 3 cm. in diameter, 500 ml.; flow rate 30 ml./hr.). The protein fraction was collected by a fraction collector and the degree of casein digestion was measured.

From each of the two original fractions, three protein fractions were obtained after the eluting started. The activity was observed in the third fraction which was eluted while the pH of the eluted solution was dropping from 5.0 to 4.0. The third fraction was collected, then dialyzed with water and thereafter freeze-dried. The purified preparation obtained from the first fraction was an almost pure acid protease preparation showing a homogeneous peak through ultracentrifugal analysis; it was dissolved in a small amount of water, followed by the addition of acetone to make the concentration about 30 percent, and the mixture was held in a refrigerator to obtain square boardlike crude crystals. The yield of the active enzyme after purification with DEAE-cellulose, in terms of the degree of casein digestion, was 67 percent in the case of the first fraction and 6.2 percent in the second fraction based on the crude enzyme powder.

To remove some slight contamination of impure substances in the purified enzyme fractions obtained, the two purified fractions were again chromatographed on the column of DEAE-cellulose and subsequently developed on the column of DEAE-cellulose by the method of gradient elution on ionic strength at pH 4.0. The purified enzyme solutions collected were concentrated under cold conditions and then crystallized by the similar method as above cited.

In the purified crystalline protease obtained, the same enzymological characteristics as that stated hereinbefore were represented with high activity, and the sedimentation constants (S_{20w}) of the first fraction and the second fraction were calculated from Svedberg's equation as 3.1S and 3.4S respectively.

EXAMPLE 4

750 g. of wheat bran was suspended in 4 liters of water, then sterilized in the same way as in example 2, thereafter transfused into a presterilized small-scale fermentation tank, followed by the addition of a solution containing monopotassium phosphate 7.5 g. and calcium chloride 0.75 g. The pH being then adjusted to 4.0, the volume of the liquid being made 14 liters, the liquid was sterilized by steaming for 1 hour under a pressure of 2 kg./cm². The liquid was then cooled to 28° C., and, thereafter, 1 liter of a spore suspension of *Penicillium frequentans* 7–5 or *Penicillium frequentans* 47–4 prepared by the method shown in example 2 was inoculated. The organism was allowed to grow under submerged cultivation conditions for 50 hours at an agitation rate of 400 r.p.m. and a rate of aeration of 10–15 liters/min., at 28° C. The activity of the protease after completion of cultivation was 1,110 tyrosine γ/ml. in the case of the former strain in terms of the degree of casein digestion at pH 2.7, the amount of acid protease accumulated being strikingly large, whereas, in the case of the latter strain, it was 230 tyrosine γ/ml.

After completion of cultivation, the culture liquid was salted out with ammonium sulfate by the same process as that stated in example 3, thereafter desalted and freeze-dried, thereby preparing crude enzyme powder. The yield of the active enzyme at the time of purification was about 30 percent in the case of the former strain and, also, about 30 percent even in the case of the latter strain in terms of the degree of casein digestion. The crude enzyme preparations of both had a pH similar to the optimum pH for casein shown by the crude enzyme preparation from *Penicillium spinulosum* T–4 and acted in the same way on collagen, this function being somewhat superior in the preparation from the latter strain.

EXAMPLE 5

1 liter of the spore suspension of *Penicillium citreo-viride* 36–7 prepared in the same manner as in example 4 was inoculated into 14 liters of the medium prepared in the same way as in example 4, and subjected to aerobic submerged cultivation for 50 hours at 28° C. under the same conditions. The activity of the acid protease after completion of cultivation was 950 tyrosine γ/ml. in terms of the degree of casein digestion, the amount of enzyme accumulated being large.

The culture liquid was purified in the same manner as in example 3, that is to say, calcium acetate was added as a precipitant and the enzyme thereafter fractionated by the addition of ammonium sulfate. The yield of active crude enzyme was 50 to 70 percent. The preparation showed an optimum pH for digesting casein between 2.2 and 2.4 and was also able to solu-

bilize collagen.

In accordance with this invention the solubilization of insoluble collagen can be carried out by using the above-described enzymes in an acid medium at a pH of about 1.5 to 3.0. The digestion medium may be acidified with common inorganic acids such as hydrochloric, sulfuric or phosphoric acids or with organic acids such as citric or acetic acid. It is known that certain acids, most notably formic acid, will cause denaturation of the collagen and such acids, for that reason, should be avoided.

The amount of the enzyme which is required in commercial process will depend on the activity thereof. In general it may be stated that the amount of enzyme should be between about 0.01 percent and 1.0 percent by weight of the collagen.

In order to recover the solubilized collagen in a fiber reconstitutable form, the temperature of the process should be below about 37° C. at all times during the preparation of the hides and digestion steps. To provide an adequate margin of safety, the temperature should generally be below about 30° C., preferably about 25° C. For practical purposes the digestion process does not proceed at economical rates at temperatures below about 0° C.

The solubilization of collagen in accordance with this invention is illustrated by the following examples.

EXAMPLE 6

3 kg. of fresh adult hide (containing 1.3 kg. of insoluble collagen) immediately after slaughtering was washed with water and then with a 5 percent saline solution to remove the water-soluble proteins such as albumin, grobulin, etc., and thereafter the hide was unhaired by enzyme treatment and washed further with water. The hide so treated was cut into pieces of about 10 cm.², followed by the addition of 20 l. of hydrochloric acid having a pH of 2, and the resulting mixture was left alone with occasional agitation. During this period, hydrochloric acid was added from time to time to maintain the pH at 2.4. When the pH remained constant the hide was minced by using a mincer with care so that the temperature of the minced hide did not exceed 30° C.; the minced hide was then collected and placed in a stirrer of high efficiency, followed by the addition of hydrochloric acid having a pH in the range of from 2 to 2.5 to the stirrer to make the total weight 20 kg. The resulting aqueous dispersion contains only swollen collagen fibers and no other proteinaceous materials. While stirring, 2.5 g. of a proteolytic enzyme, which is produced from *Penicillium spinulosum* T-4 (A.T.C.C. No. 16348) by the solid cultivation method mentioned above, was added after being dissolved in a small amount of dilute hydrochloric acid. The resulting mixture, while being kept at a temperature of about 25° C., was agitated constantly for 24 hours to solubilize 96 percent of the insoluble collagen of the hide, and was thereafter continuously agitated for an addition 12 hours to obtain a highly viscous solution. The viscous solution was filtered by using a filter press. The nitrogen content of the filtrate, when measured by the Kjeldahl method, showed that the filtrate container collagen in a concentration of about 6.5 percent w/v, and, when examined by a physicochemical method, contained collagen molecules having a length of about 3,000 A, a molecular weight of about 260,000 and a rigid, rod helical structure. This shows that the collagen in the solution has maintained its native collagen structure.

The collagen fibers were reconstituted with a yield of nearly 100 percent from the filtrate by neutralizing it or by dialyzing it with an aqueous solution of disodium phosphate, and yet, when examined by an electron microscope, the reconstituted collagen fibers were found to have the striation of periodicity of 700 A peculiar to native collagen fibers.

EXAMPLE 7

The procedure described in example 1 was followed using a proteolytic enzyme produced from *Penicillium frequentans* 47-4. (A.T.C.C. No. 16349) by the above-mentioned aeration-stirring submerged cultivation method, in place of the enzyme used in example 1. In this procedure, the insoluble collagen was completely solubilized at a temperature of about 25° C. in 36 hours after the addition of the enzyme, and the collagen obtained had the same characteristics as those of the collagen obtained by the procedure in example 1.

EXAMPLE 8

The procedure described in example 1 was followed using a proteolytic enzyme produced from *Penicillium citreo-viride* 36-7 (A.T.C.C. No. 16351) by the above-mentioned aeration-stirring submerged cultivation method, in place of the enzyme used in example 1. In this procedure, about 95 percent of the insoluble collagen was solubilized at a temperature of about 25° C. in 24 hours after the addition of the enzyme, and after agitation for another 12 hours, the collagen was completely solubilized. The collagen obtained had the same characteristics as those of the collagen obtained by the procedure in example 1. The disclosures of the related applications cited hereinbefore are to be included herein by reference.

We claim:

1. In a method of solubilizing insoluble collagen wherein insoluble collagen is treated with an enzyme at a temperature of 0°~37° C. and the enzyme-treated product is dissolved into an acid, the improvement comprising treating said insoluble collagen at a pH of 1.5 to 3.0 with the collagen-solubilizing proteolytic enzyme produced from a micro-organism selected from the group consisting of *Penicillium spinulosum* ATCC No. 16,348, *Penicillium frequentans* ATCC Nos. 16,349 and 16,350, and *Penicillium citreo-viride* ATCC No. 16,351, having an optimum activity for solubilizing milk casein at a pH of 2.2 to 2.4.

2. A method according to claim 1 wherein said enzyme is produced by *Penicillium frequentans*, ATCC No. 16,349.

3. A method according to claim 1 wherein said enzyme is produced by *Penicillium frequentans*, ATCC No. 16,350.

4. The method according to claim 1 wherein said enzyme is produced by *Penicillium spinulosum*, ATCC No. 16,348.

5. The method according to claim 1 wherein said enzyme is produced by *Penicillium citreo-viride*, ATCC No. 16,351.

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