

1.185.335

PATENT SPECIFICATION

DRAWINGS ATTACHED

1.185.335



Inventors: MASAO ISONO, KATSUMI TOMODA, KOICHI MIYATA, KAZUTAKA MAEJIMA and REIJIRO KODAMA

Date of Application (No. 24849/67) and filing Complete Specification: 30 May, 1967.

Complete Specification Published: 25 March, 1970.

Index at acceptance:—C3 H3; A5 B(31Y, 312, 38Y, 381)

International Classification:—C 12 d 13/10

COMPLETE SPECIFICATION

A method for producing Protease

We, TAKEDA YAKUHI KOGYO KABUSHIKI KAISHA (TAKEDA CHEMICAL INDUSTRIES LTD.) of 27, Doshomachi 2-chome, Higashi-ku, Osaka, Japan, a corporation organised under the laws of Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to a protease which shows strong caseinolytic and fibrinolytic activities and which is useful as an anti-inflammatory agent, and to a process for producing the protease in a form which is pure and highly active enough to be used for the intended purpose set forth below. This invention also relates to a novel use of the protease as an anti-inflammatory agent.

We have found that a bacterium which had been isolated by us and named as *Serratia* sp. E-15 produces a protease which has a strong proteolytic activity. Our further study revealed that the protease-productivity of E-15 is remarkably enhanced under specific conditions. As a result, it has become possible to obtain the protease in a highly purified form, so that the protease can be used for medical purposes as well as for the study of its enzymic properties. The availability of the purified protease produced by E-15 contributed to our other finding that the protease shows an unusually high caseinolytic and fibrinolytic activities and that it can be administered to animals (including human beings) without any significant ill effect and without being inactivated, and that the protease exhibits an anti-inflammatory effect, much stronger than that of known anti-inflammatory agents such as phenylbutazone.

The present invention provides a protease which is sufficiently highly purified to be substantially free from any hazards for its novel use as an anti-inflammatory agent; a specific process for producing the protease abundantly and easily, an anti-inflammatory composition

comprising this protease, and a method for treating inflammation in animals excluding humans with this composition.

In order to realise the objects of the present invention, a bacterium, *Serratia* sp. E-15 or its mutant or variant is cultivated in a culture medium.

The pharmaceutical composition of the invention may comprise a major proportion or a minor proportion of the protease and a minor proportion or a major proportion of a pharmaceutically acceptable carrier therefor.

We isolated *Serratia* sp. from a silkworm intestine, and it showed the following microbiological properties:

Microbiological properties of Serratia sp. E-15

Short rods 0.8 to 1.0 by 1.2 to 1.8 microns. Endospore not formed. Motile by means of peritrichous flagella. Not acidfast. Gram-negative.

Agar colonies: Smooth, usually circular, grayish white. Pink pigment may diffuse in the medium, if produced.

Broth: Rapidly turbid. Liquid becomes pink after prolonged cultivation.

Gelatin stab: Rapid stratiform liquefaction.

Milk: Coagulation. Peptonisation.

Casein: hydrolysed.

Esculin: hydrolysed.

Urea: hydrolysed.

Starch: not hydrolysed.

Arginine: hydrolysed.

Nitrites: produced from nitrates.

Hydrogen sulphide: not produced.

Indole: not formed.

Ammonia and trimethylamine: produced.

Voges-Proskauer test: positive.

Methyl red test: faint.

Citrates: utilised as sole source of carbon.

Ammonium salts: not utilised as sole source of nitrogen.

Methylene blue dye: reduced.

Cytochrome oxidase reaction: negative. Grows on KCN-media.

[Price 5s. 0d.]

Acid produced from maltose, sucrose, cellobiose, trehalose, glucose, fructose, mannose, galactose, ribose, calicin, glycerol, mannitol, adonitol and inositol.

5 Acid not produced from starch, dextrin, inulin, raffinose, lactose, melibiose, melesitose, sorbose, rhamnose, arabinose, xylose, methylglucoside and dulcitol.

Optimal temperature for growth: 30°C. Does not grow at 45°C.

Salt tolerance: grows in 7.5% NaCl. Does not grow in 8% NaCl. Catalase positive.

Distinctive characters: Pigment from this organism reveals a characteristic spectrum of prodigiosin which has an absorption maximum at 540 mμ wavelength. The pigment is freely soluble in ethanol, methanol and water.

According to Bergey's "Manual of Determinative Bacteriology" 7th edition (1957), these microbiological properties indicate that the bacterium E-15 belongs to:

the class *Schizomycetes*
the order *Eubacteriales*
the family *Enterobacteriaceae*
25 the tribe *Serratiae*
the genus *Serratia*

Although E-15 most resembles *Serratia piscatorium* among the species presented in Bergey's Manual as belonging to the genus *Serratia*, E-15 cannot be designated *S. piscatorium*, because *S. piscatorium* does not hydrolyse urea and produces pigment within 8-hour cultivation, while E-15 does hydrolyse urea and does not produce pigment within 8-hour cultivation. Moreover, the pigment produced by E-15 is quite different from that of *S. piscatorium* in its solubility in water. Therefore, the strain E-15 must be defined as constituting a new species of the genus *Serratia*, and is expressed in this specification as "*Serratia* sp. E-15", or simply "E-15".

It is well known as a general conception on microorganisms that microbiological characteristics are not generally fixed. This is true of *Serratia* sp. E-15 and many natural or induced mutants or variants can be derived from *Serratia* sp. E-15. Artificial induction of the mutation of variation can be effected by conventional procedures, for example, by irradiating *Serratia* sp. E-15 with ultraviolet rays or X-rays, or by treating the microorganism with a chemical compound such as nitrogen mustard or magnesium chloride. These mutants or variants are also used for the present invention so far as they retain the originally endowed ability to produce protease. Therefore, it should be understood that, hereinafter, in the general description of this specification, the reference "E-15" includes these mutants and variants.

A specimen of *Serratia* sp. E-15 has been deposited at the American Type Culture Collection, Maryland, U.S.A. under the accession number ATCC 21074.

In order to cultivate E-15, conventional culture media for *serratia* can be used. However, the protease productivity of E-15 is greatly affected by the constituents of the culture media, especially by the organic nitrogen source. The protease can be produced in a culture medium which comprises any of the assimilable nitrogen sources such as nitrates, cornsteep liquor, peptone, casein, meat extract and soybean meal, which may be supplemented by ammonium salts. However, in view of the resulting protease productivity of E-15, co-employment of milk casein and soybean meal extract results in a striking increase in the protease productivity which enables us to obtain a very active and purified protease abundantly. The synergetic effect of the co-employment of milk casein and soybean meal extract is specific, and the effect is not observed any more in other combinations.

Soybean meal extract is prepared by extracting soybean meal or defatted soybean meal with water at room temperature (about 20°C. to 25°C.) for about 30 minutes with stirring, and by filtering off the insolubles. In the present invention, "X % (weight per volume) of soybean meal extract" means a product which is prepared by adding X part(s) by weight of soybean meal or defatted soybean meal to 100 parts by volume of water, stirring the mixture at room temperature for about 30 minutes, and subjecting the treated mixture to filtration to remove the insolubles therein. The following tests will show how much of the milk casein and soybean meal extract should be used for advantageous production of the protease. In this specification, percentages of culture medium constituents are weight per volume values based on the medium. Parts by weight bear the same relation to parts by volume as do grams to millilitres.

TEST 1

Ten culture media (A) to (J) were prepared by adding 2% of milk casein and a varied amount of soybean meal extract as illustrated in Table 1 to a basic culture medium (pH 7.0) which consists of 1% of ammonium phosphate, 0.05% of potassium chloride, 0.1% of sodium chloride, 0.02% of calcium chloride (2 hydrate), 0.02% of magnesium sulphate (7 hydrate) and water. *Serratia* sp. E-15 was cultivated in each of the 10 culture media at 28°C. for 2 days by the use of a rotary shaker. The respective culture broths were centrifuged to remove cells, and the protease activity of each supernatant solution was measured by the Kunitz method (J. Gen. Physiol. 30, 291, 1947) under the conditions of pH 9 at 37°C. to give the results shown in Table 1.

One protease unit (PU) was defined as the enzyme amount producing 1 μg equivalent tyrosine per minute.

TABLE 1

Culture Medium	Amount of Soybean Meal Extract (%)	Protease Activity (PU/ml)
A	0	672
B	0.5	1,460
C	1.0	3,750
D	2.0	4,170
E	5.0	4,740
F	10.0	4,370
G	15.0	3,500
H	20.0	2,880
I	25.0	2,180
J	30.0	2,010

TEST 2

Seven culture media (K) to (Q) were prepared by adding 2% of soybean meal extract and a varied amount of milk casein as illus-

trated in Table 2 to the basic culture medium which has the same composition as in Test 1. The same procedure as in Test 1 gave the result shown in Table 2.

TABLE 2

Culture Medium	Amount of Milk Casein (%)	Protease Activity (PU/ml)
K	0	928
L	0.5	1,570
M	1.0	3,750
N	2.0	4,230
O	5.0	4,250
P	10.0	4,330
Q	15.0	2,120

The synergistic effect between milk casein and soybean meal extract is obvious from the result of Tests 1 and 2, and it is most important when the respective concentrations of milk casein and soybean meal extract in the culture medium are from about 1 to about 10 percent and from about 1 to about 15 percent.

This synergistic effect is so specific that it is not observed when other nitrogen sources than milk casein are used together with soybean meal extract, as shown in test 3.

TEST 3

1% of soybean meal extract was added to an aqueous culture medium which has the same composition as that of the basic medium used in Test 1. The mixture was divided into six equal portions. One was used for control, and the other five portions were used in the test after 1% of an additional nitrogen source, i.e. cornsteep liquor, gelatine, casein hydrolysate (casamino acid), peptone or milk casein had been added. The same procedures for cultivation and protease activity assay as in Test 1 gave the results shown in Table 3.

TABLE 3

Additional Nitrogen Source	Protease Activity (PU/ml)
None	670
Cornsteep liquor	505
Gelatin	1,090
Casein hydrolysate	1,240
Peptone	1,880
Milk casein	3,660

So far as both milk casein and soybean meal extract in the optimum amounts are used in the culture medium, it is not necessary to use any other particular carbon sources. It is rather recommended not to use certain carbon sources such as glucose, sucrose, maltose, or dextrin, because the addition of such carbon sources will acidify the culture medium and the acidified medium will provide undesirable conditions for producing highly active protease.

Other nutrients such as metal salts, phosphates, vitamins and growth factors are usually added to the medium. So far as such additional nutrients are concerned, E—15 can grow on a culture medium which contains ordinary nutrients for the cultivation of *Serratia*. However, it is recommended to use a medium containing calcium chloride and phosphates such as sodium phosphate, potassium phosphate and ammonium phosphate, for the purpose of the present protease production. The preferred amount of calcium chloride is from 0.01% to 0.05%, especially about 0.02%, in terms of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and that of the phosphate is from 0.7% to 1.5% in terms of PO_4 .

The cultivation of E—15 is carried out under aerobic conditions. In practice, it is preferred to cultivate E—15 in an aqueous medium with shaking or in a tank with stirring and aeration. The preferred aeration rate is usually from 0.5 to 1 liter per minute per liter of the aqueous medium.

Although other specific cultivation conditions should be determined mutually so as to

give the highest production of the protease, it is usual during the cultivation to adjust the temperature to from 25°C. to 35°C. and the pH of the medium to a range around the neutral point (pH 6 to pH 9), especially 7. The maximum production of the protease is usually seen after 15 to 40 hours cultivation.

The protease is thus produced by E—15 during the cultivation and is accumulated in the culture medium. When the protease activity in the medium is higher than 3,000 PU/ml, isolation of the protease is very easy and the pure protease obtained is highly active. For separating and purifying the protease, any of the known means for separation and purification which are commonly used in this field are applied, such as precipitation by the use of a precipitation aid, precipitation at the isoelectric point, salting out, dialysis, ion-exchange chromatography and gel filtration.

EXAMPLE "A"

Serratia E—15 (ATCC 21074) is inoculated in 30,000 parts by volume of a culture medium (pH 7) consisting of 1% of milk casein, 1% of soybean meal extract, 1% of $(\text{NH}_4)_2\text{HPO}_4$, 0.1% of NaCl , 0.05% of KCl , 0.02% of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3% of soybean oil and water. The culture is incubated at 28°C. for 30 hours with stirring and aeration at a rate of 0.5 liter/minute/liter of the medium. The protease activity of the culture supernatant at various stages during the cultivation is shown in Table 4.

TABLE 4

Cultivation Time (hrs.)	pH of Culture Medium	Protease Activity (PU/ml)
0	7.09	—
6	6.92	242
12	6.85	2,640
18	6.90	3,370
24	6.72	4,060
30	6.50	3,740

After 24 hours cultivation, 5,000 parts by volume of the culture broth are taken and centrifuged to obtain 4,800 parts by volume of supernatant liquor, and $(\text{NH}_4)_2\text{SO}_4$ is added up to 66% saturation to give precipitates. The resulting precipitates are collected, dissolved in 100 parts by volume of water and subjected to dialysis through a cellophane membrane ("Visking" (trade mark), Visking Co., U.S.A.) against distilled water at 5°C. for 2 days. 400 parts by volume of the inside solution so obtained are subjected to fractional precipitation by the step-wise addition of acetone, and the fraction that precipitates between 40% and 60% by volume of acetone is collected by centrifuging and dried to obtain a crude enzyme preparation.

Three parts by weight of the crude enzyme preparation are dissolved in 100 parts by volume of distilled water, and the solution is subjected to dialysis through Visking against a 0.01M phosphate buffer solution of pH 7.0 at 5°C. for 2 days. 120 parts by volume of the dialysed inside solution are allowed to flow through the column (3 × 130 cm) packed with DEAE-cellulose which is previously buffered with a 0.01M phosphate buffer solution of pH 7.0, whereupon the enzyme is adsorbed on the column. The enzyme is eluted with a 0.1M aqueous sodium chloride solution and the eluate is fractionated in 10 parts by volume portions. $(\text{NH}_4)_2\text{SO}_4$ is added to 120 parts by volume of the enzyme-rich fractions, up to 66% saturation to give precipitates. The precipitates are collected and dissolved in 30 parts by volume of a 0.01 M phosphate buffer solution of pH 7.0. The enzyme solution is subjected to gel-filtration through a column of Sephadex (trade mark) G-75 (3 × 40 cm), followed by precipitation again with 66% saturation of $(\text{NH}_4)_2\text{SO}_4$. The resulting precipitates are again dialysed through Visking against distilled water at 5°C. for 3 days. 55 parts by volume of the inside solution are lyophilised to give about 1 part by weight of

purified protease, the protease activity of which is 4,400 PU/mg.

The protease of the present invention as obtained in the Example, shows the following properties:

- (1) The enzyme exhibits the nature of a uniform and single protein when observed by centrifuging analysis. The sedimentation constant (S_{20w}) measured by an ultracentrifuge is 3.8 S, from which the molecular weight is determined to be about 6×10^4 according to the Archibald method (cf. W. J. Archibald: J. Phys. & Colloid Chem., 51, 1204, 1947.).
- (2) The elementary analysis:
C: 47.39%, H: 7.18% N: 15.18%.
- (3) The ultraviolet absorption spectrum is shown in Fig. 1, which indicates that the curve is of a typical protein which has the maximum absorption at the wave-length of 275 to 278 mμ and a shoulder at 290 mμ.
- (4) The infra-red absorption spectrum is shown in Fig. 2, and shows significant absorption bands at the following wave-lengths in microns: 3.0 (strong), 3.38 (middle), 6.05 (broad, strong), 6.55 (broad, strong), 6.88 (weak), 7.15 (broad, middle), 8.12 (middle) and 9.3 (broad, weak).
- (5) The isoelectric point measured by paper electrophoresis is between pH 5 and 6.
- (6) The optimum enzyme activity is exhibited at a pH of from 8.5 to 9.5 as shown in Fig. 3 and at a temperature of from 35°C. to 45°C., especially around 40°C., as shown in Fig. 4.
- (7) The pH stability is shown in Fig. 5, in which the solid line shows the stability at 37°C. and the broken line shows the stability at 4°C., from which it is observed that the enzyme is stable at a pH between 5 and 7 at 37°C., and is still stable even at pH 10 at 4°C.
- (8) The thermostability when measured by

- heating the enzyme solution for 10 minutes without substrate is shown in Fig. 6, which shows that the enzyme is stable at 40°C but the activity is lost by 10 minutes at 60°C.
- (9) The enzyme activity is inhibited in the presence of Ni^{++} , Cd^{++} , Cu^{++} or Hg^{++} .
- (10) The enzyme activity is inhibited in the presence of ethylenediamine tetraacetate at the pH from 4 to 6 as shown in Fig. 7, but the activity is restored by the addition of Zn^{++} , Mn^{++} or Co^{++} .
- (11) The enzyme activity is not inhibited in the presence of *p*-chloromercuribenzoates or diisopropyl fluorophosphate.
- (12) The protease obtained is highly active in its proteolytic activity, especially in its caseinolytic and fibrinolytic activities, the caseinolytic activity being higher than 2,000 PU/mg. In comparison with known alkaline or neutral proteases, the protease of this invention is stronger in its proteolytic activity, as shown in Tables 5 and 6.

TABLE 5

Enzyme	Caseinolytic Activity (PU/mg)	
	pH 7.1	pH 9.0
E-15 protease	2,100	3,612
Chymotrypsin	611	688
Bromelin	106	104
Pronase	418	710

TABLE 6

Enzyme	Amount of Enzyme Required for Dissolving fibrin within 10 minutes
E-15 protease	4 $\mu\text{g}/\text{ml}$
Chymotrypsin	50 $\mu\text{g}/\text{ml}$
Bromelin	>100 $\mu\text{g}/\text{ml}$
Pronase	50 $\mu\text{g}/\text{ml}$

In view of the stability and the optimal activity of the protease at about 37°C. in the neighbourhood of neutral pH, the protease of this invention is used as an effective digestive or anti-inflammatory agent. The following test is a comparison test between the protease of the present invention and phenylbutazone in their anti-inflammatory effects.

TEST 4

The illustrated amount of test samples was injected intraperitoneally into female rats weighing 120 to 150 grams. Thirty minutes

after, an irritant was injected into a hind paw of the rats as well as of those rats as a control to which no test compounds had been administered. The capacity of the swelling due to the irritant was then measured every hour. Inhibitory activities to the inflammatory oedema caused by the irritants, i.e. (1) 0.05 ml of 1% aqueous carrageenin solution, (2) 0.05 ml of 6% aqueous dextran solution and (3) 0.05 ml of 10% aqueous ovalbumin solution, are shown in Table 7 in terms of swelling per cent.

TABLE 7

Irritant	Test Sample	Dosage mg/kg	Time after injection of Irritant (hrs)					
			0	1	2	3	4	5
(1)	E-15 protease (4,000 PU/mg)	5	0	20	26	23	26	24
		10	0	10	13	17	16	13
	E-15 protease (3,159 PU/mg)	20	0	15	19	20	21	20
		50	0	7	6	13	14	13
	Phenylbutazone	50	0	13	19	22	24	23
	Control	0	0	53	71	76	81	80
(2)	E-15 protease (3,159 PU/mg)	20	0	37	41	36	34	32
		50	0	16	18	17	16	15
	Phenylbutazone	50	0	58	60	54	52	50
	Control	0	0	54	76	79	80	78
(3)	E-15 protease (3,159 PU/mg)	20	0	41	42	38	35	35
		50	0	14	15	15	12	11
	Phenylbutazone	50	0	53	63	63	58	56
	Control	0	0	64	88	86	84	81

From Table 7, it is clearly seen that the protease of the present invention shows far superior anti-inflammatory and anti-swelling activity to that of phenylbutazone.

5 Acute toxicity of the protease (3157 PU/mg) in mice or rats after 72 hours from

the administration is shown in Table 8. The range in brackets shows 95% confidence limit. The values shown are LD₅₀ values in mg/kg (amounts in mg per kg of body weight to cause 50% death).

TABLE 8

Administration	Mice	Rats
Oral	No animal died at 2,500 mg/kg	No animal died at 2,000 mg/kg
Subcutaneous	44 (36.1—53.78)	—
Intraperitoneal	18.34 (14.32—23.49)	—
Intravenous	14.23 (12.45—16.26)	—

15 In order to test the subacute toxicity, the protease (3157 PU/mg) of the present invention was orally administered to rats (Wister strain), each weighing about 120 grams, every day for a month. Groups, each consisting of 15 rats, were scheduled as follows:

Group	Sex	Dose (Mg/kg/day)	Remarks
1	male	0	} Control
2	Female	0	
3	Male	3	} Dosage corresponds to 10 times as much as the usual dosage for human beings
4	Female	3	
5	Male	15	} " 50
6	Female	15	
7	Male	60	} " 120
8	Female	60	

5. In comparison with the control groups (Groups 1 and 2), none of the test groups (Groups 3 to 8) showed substantial difference in general appearance, body weight, blood liver function, blood sugar level, serum electrolytes, urine excretion and weights of viscera.

10 As shown above, the protease of this invention is substantially non-toxic, and exhibits a strong anti-inflammatory activity when administered orally.

15 Unlike hitherto-known steroidal anti-inflammatory agents which often cause metabolic disturbance due to pituitary and adrenal abnormality, the protease of this invention acts directly on the inflammation without affecting the pituitary and adrenal systems, so that it provides a safer countermeasure against inflammation than the known agents.

Moreover, the protease of this invention is effectively absorbed through the intestine wall so that it is easily administered *per os*. However, in order to avoid the affection of acid gastric juice, it is recommended that the protease should be administered *per os* in the form of an enteric-coated preparation. When the protease is administered orally, the usual daily dose for a human adult is from 5 to 60, preferably, from 15 to 30 milligrams of the enzyme which has a protease activity higher than 2,000 PU/mg.

For the purpose of giving those skilled in the art a better understanding of the invention, the following illustrative examples of preparation are given.

I. A capsule filled with enteric granules.

Capsule prescription (per capsule);

Protease of the present invention	5 mg. (10,000 PU)
Lactose	46 mg.
Corn starch	16 mg.
Crystalline cellulose	12 mg.
Cellulose glycollic acid	5 mg.
Sorbitol	10 mg.
	100 mg.

II. An enteric tablet:
Tablet prescription (per tablet);

Protease of the present invention	5 mg. (10,000 PU)
Lactose	79 mg.
Corn starch	45 mg.
Gelatine	0.5 mg.
Magnesium stearate	0.5 mg.
	130 mg.

WHAT WE CLAIM IS:—

1. A method for producing a protease, which comprises cultivating *Serratia sp.* E—15 (ATCC 21074) or a mutant or variant thereof, in an aqueous culture medium containing from 1% to 10% (weight per volume) of milk casein, from 1% to 15% (weight per volume) of soybean meal extract and other nutrients necessary for the growth of the microorganism at a temperature of from 25°C. to 35°C., under aerobic conditions, until the protease is substantially accumulated in the culture broth, and separating the accumulated protease from the liquid part of the culture broth.
2. A method according to claim 1, wherein the cultivation is carried out until the protease activity of the medium has become higher than 3,000 PU/ml.
3. A method according to claim 1, wherein the aqueous culture medium contains from 0.01% to 0.05% (weight per volume) of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.
4. A method according to claim 1, wherein the aqueous culture medium contains from 0.7% to about 1.5% (weight per volume), in terms of PO_4 , of phosphate.
5. A method as claimed in claim 1 substantially as herein described.
6. A method as claimed in claim 1 substantially as herein described with reference to the specific example "A".
7. A protease when produced by a method as claimed in any of claims 1 to 6.
8. A unitary protease having the properties herein before defined.
9. A pharmaceutical composition, which comprises, as active ingredient, a major proportion of a unitary protease as claimed in claim 7 or 8, together with a minor proportion of a pharmaceutically acceptable carrier therefor.
10. A pharmaceutical composition, which comprises, as an active ingredient, a minor proportion of a unitary protease as claimed in claim 7 or 8, together with a major proportion of a pharmaceutically acceptable carrier therefor.
11. A pharmaceutical composition as claimed in claim 9 or 10, substantially as herein described.
12. A pharmaceutical proposition as claimed in claim 9 or 10 substantially as herein described in with reference to either of the specific examples I and II.
13. A method for treating inflammation in non-human animals, which comprises administering to such an animal having an inflammation from 5 to 150 milligrams per day of a protease or a composition as claimed in any of claims 7 to 12.
14. A method as claimed in claim 13 substantially as herein described.

ELKINGTON AND FIFE,
Chartered Patent Agents,
High Holborn House,
52/54 High Holborn,
London, W.C.1.
Agents for the Applicants.

II. An enteric tablet:
Tablet prescription (per tablet);

Protease of the present invention	5 mg. (10,000 PU)
Lactose	79 mg.
Corn starch	45 mg.
Gelatine	0.5 mg.
Magnesium stearate	0.5 mg.
	130 mg.

WHAT WE CLAIM IS:—

1. A method for producing a protease, which comprises cultivating *Serratia* sp. E—15 (ATCC 21074) or a mutant or variant thereof, in an aqueous culture medium containing from 1% to 10% (weight per volume) of milk casein, from 1% to 15% (weight per volume) of soybean meal extract and other nutrients necessary for the growth of the microorganism at a temperature of from 25°C. to 35°C., under aerobic conditions, until the protease is substantially accumulated in the culture broth, and separating the accumulated protease from the liquid part of the culture broth.

2. A method according to claim 1, wherein the cultivation is carried out until the protease activity of the medium has become higher than 3,000 PU/ml.

3. A method according to claim 1, wherein the aqueous culture medium contains from 0.01% to 0.05% (weight per volume) of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.

4. A method according to claim 1, wherein the aqueous culture medium contains from 0.7% to about 1.5% (weight per volume), in terms of PO_4 , of phosphate.

5. A method as claimed in claim 1 substantially as herein described.

6. A method as claimed in claim 1 substantially as herein described with reference to the specific example "A".

7. A protease when produced by a method as claimed in any of claims 1 to 6.

8. A unitary protease having the properties herein before defined.

9. A pharmaceutical composition, which comprises, as active ingredient, a major proportion of a unitary protease as claimed in claim 7 or 8, together with a minor proportion of a pharmaceutically acceptable carrier therefor.

10. A pharmaceutical composition, which comprises, as an active ingredient, a minor proportion of a unitary protease as claimed in claim 7 or 8, together with a major proportion of a pharmaceutically acceptable carrier therefor.

11. A pharmaceutical composition as claimed in claim 9 or 10, substantially as herein described.

12. A pharmaceutical proposition as claimed in claim 9 or 10 substantially as herein described in with reference to either of the specific examples I and II.

13. A method for treating inflammation in non-human animals, which comprises administering to such an animal having an inflammation from 5 to 150 milligrams per day of a protease or a composition as claimed in any of claims 7 to 12.

14. A method as claimed in claim 13 substantially as herein described.

ELKINGTON AND FIFE,
Chartered Patent Agents,
High Holborn House,
52/54 High Holborn,
London, W.C.1.
Agents for the Applicants.