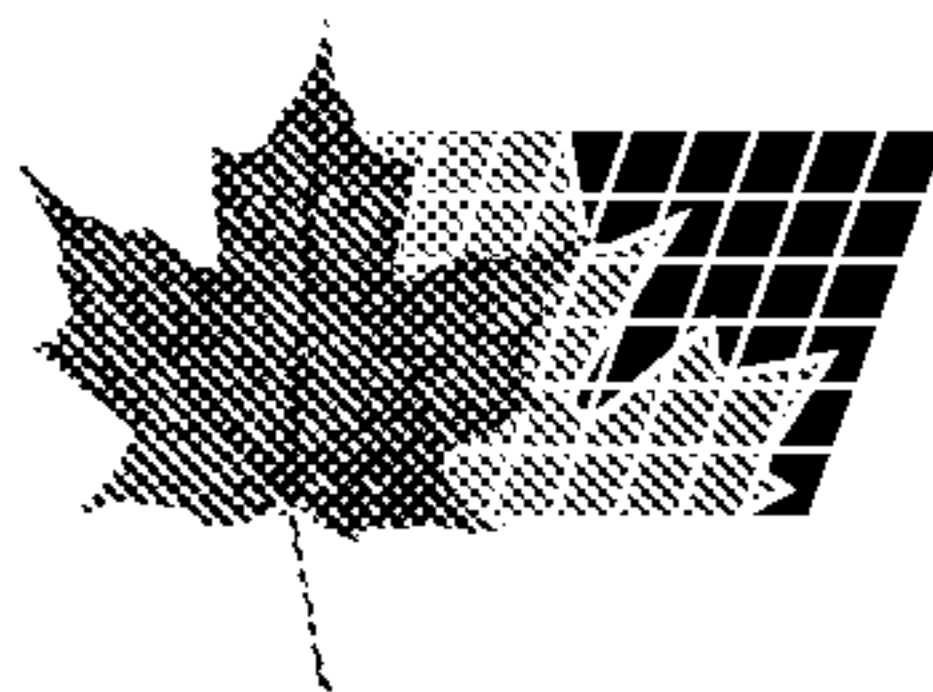




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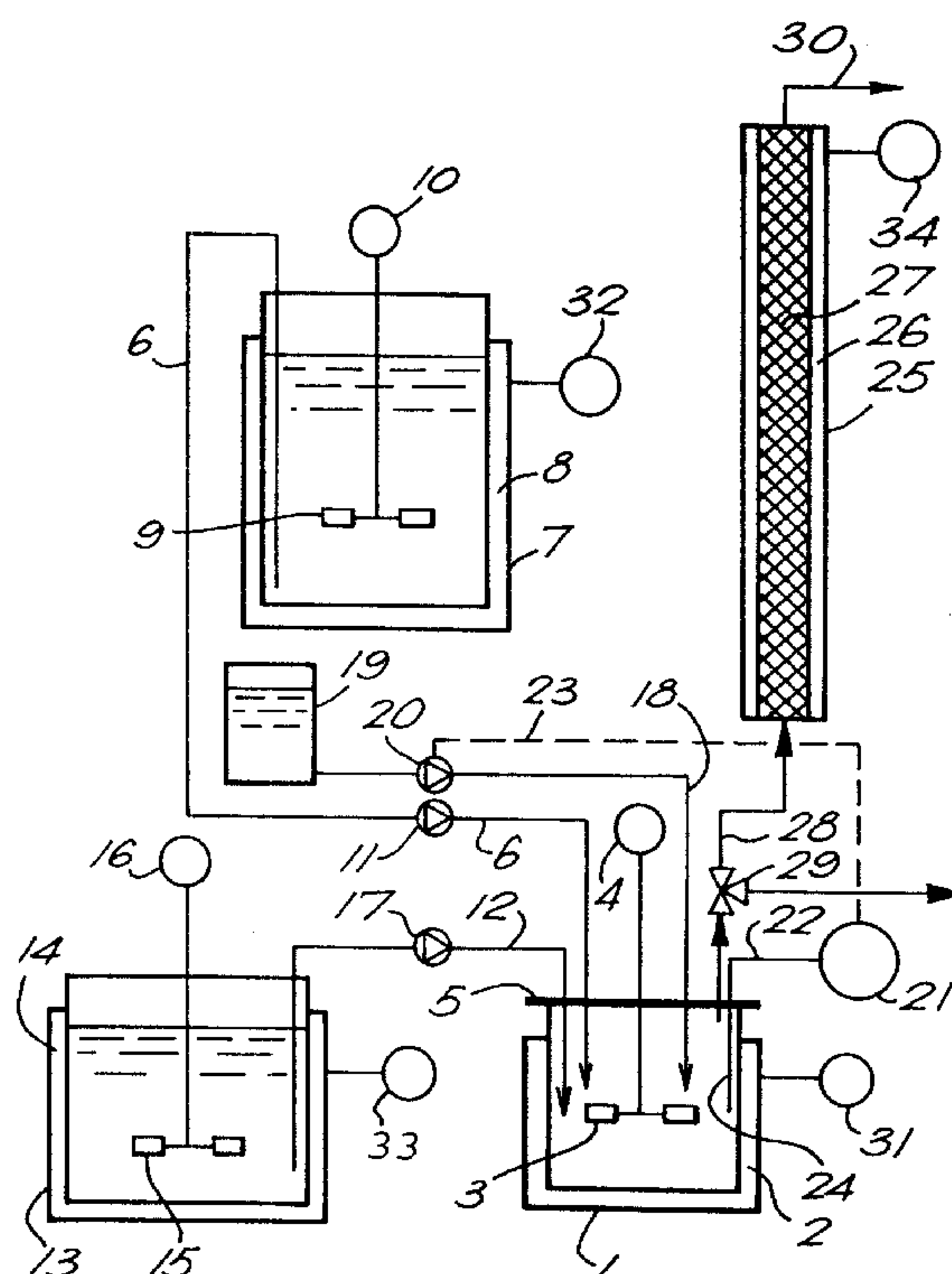
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(54) **HYDROLYSE ENZYMATIQUE**

(54) **ENZYMATIC HYDROLYSIS**



(57) A process and apparatus for the enzymatic hydrolysis of proteins, in which a proteolytic enzyme and a protein substrate are mixed, a first hydrolysis step is carried out in a stirred tank and a second hydrolysis step is carried out in a tube equipped with static mixing elements.



ABSTRACT

A process and apparatus for the enzymatic hydrolysis of proteins, in which a proteolytic enzyme and a protein substrate are mixed, a first hydrolysis step is carried out in a stirred tank and a second hydrolysis step is carried out in a tube equipped with static mixing elements.

This invention relates to a process for the enzymatic hydrolysis of proteins and to an apparatus for carrying out this process.

5           There are various known processes for the enzymatic hydrolysis of proteins which differ from one another, for example, in the choice of the substrate, the enzyme, the degree of hydrolysis and/or the required peptide profile. In cases where, for example, a relatively well-defined peptide profile, more particularly  
10           a narrow oligopeptide profile, is required for reasons of assimilation of the hydrolyzate by the intestinal mucosa, known hydrolysis processes generally comprise at least one hydrolyzate filtration or screening step.

15           For example, EP 226 221 describes a process for the preparation of hypoallergenic peptides having a molecular weight in the range from 2000 to 6000 by one or more enzymatic protein hydrolysis steps each carried out discontinuously in a fermentation tank and each  
20           terminating in an ultrafiltration step.

          US 4,212,889 describes a process for solubilizing fish proteins, in which a mixture of fish flesh and enzyme is continuously passed through an installation comprising several hydrolysis tanks connected in series.

25           The problem addressed by the present invention was to provide a hydrolysis process which, preferably carried out continuously, would enable the efficiency of discontinuous hydrolysis in a tank to be equalled or even increased and which would enable a protein hydrolyzate having a well-defined and reproducible degree of  
30           hydrolysis and/or peptide spectrum to be obtained.

          To this end, the process according to the invention for the enzymatic hydrolysis of proteins, in which a protein substrate is subjected to hydrolysis with a  
35           proteolytic enzyme, comprises a first enzymatic hydroly-

sis step in a stirred tank and a second enzymatic hydrolysis step in a tube. In a preferred embodiment, the process according to the invention is carried out continuously and the second enzymatic hydrolysis step is  
5 carried out in a tube equipped with static mixing elements.

Similarly, the apparatus for carrying out the process according to the invention comprises a double-jacketed stirred hydrolysis tank which is connected  
10 upstream to a substrate metering unit and to an enzyme metering unit and downstream to at least one hydrolysis tube. In a preferred embodiment, the tube is equipped with static mixing elements.

It has been found that it is thus possible to  
15 produce a protein hydrolyzate having a well-defined degree of hydrolysis and/or peptide spectrum in a highly efficient and reproducible, preferably continuous manner.

By virtue of the process and apparatus according  
20 to the invention, it is possible in particular to work with a tank of relatively small dimensions which may be filled completely without leaving any head space and with a tube of relatively large dimensions. It is thus possible to carry out, preferably continuously, a first  
25 relatively short step, i.e. a hydrolysis initiation step, in a relatively small tank, and a second relatively long step, i.e. a hydrolysis completion step, in a tube of relatively large volume. The reaction time, i.e. the residence time of the substrate in the total  
30 volume represented by the sum of the tank volume and the tube volume, can thus be controlled in a precise and simple manner, for example by means of volumetric pumps.

If, for comparison, it is desired to carry out a hydrolysis in a single hydrolysis tank of large dimen-  
35 sions, the residence time of a unit volume of hydroly-

zate cannot be precisely defined. This is true of discontinuous hydrolysis where the times required to establish given pH and/or temperature conditions and even to empty the tank, for example, are considerable. However, the same is even truer of continuous hydrolysis, in which case is it only possible to define a mean residence time. Even in a process of the type described in the above-cited US 4,212,889, the residence time can scarcely be defined any more precisely.

By contrast, it has been found that, with the process and apparatus according to the present invention, the residence time of a unit volume of hydrolyzate can be defined in a remarkably precise manner, the flow of hydrolyzate through the hydrolysis tube - preferably equipped with static mixing elements - having a very flat front.

In the present specification, the degree of hydrolysis is defined via the quantity of non-protein nitrogen (NPN) determined as the percentage of total nitrogen which cannot be precipitated with 13% trichloroacetic acid.

The nitrogen contents are determined by the Kjeldahl method.

The amine nitrogen (free  $\alpha$ -NH<sub>2</sub>) contents are determined by reaction with ninhydrin after alkaline hydrolysis.

The serotonin relaxation tests using tritium-labelled exogenous serotonin (serotonin-<sup>3</sup>H) are carried out on normal mastocytes of the peritoneal cavity of rats by the method described by R. Fritsché and M. Bonzon in Int. Arch. Allerg. Immunol. 93, 289-293 (1990).

The ELISA inhibition tests are carried out with rabbit antibodies specific of  $\beta$ -lactoglobulin (BLG), bovine serum albumin (BSA) and casein (CAS). The sensi-

tivity of the method, i.e. the concentration detection limit, is 20 ng/ml.

5 The high performance liquid chromatography analyses (HPLC tests, peptide profiles) are carried out under non-denaturing conditions on gel based on type TSK-G2000-SW™ silica (a product of Toyo Soda), of which the fractionation range extends from 500 to 50,000 dalton, in a Biorad BIOSIL SEC-125™ column. The results are expressed in % surface distribution of the peaks  
10 read at 220 nm in a 0.1 M phosphate solution + 0.4 M NaCl at pH 6.80.

The analyses by zone electrophoresis in polyacrylamide gel (SDS-PAGE tests) are carried out by the method described by Laemmli in Nature 227, 680 et seq.  
15 (1970).

The blockage of lysine is determined by HPLC and is expressed as % blocked lysine relative to the total lysine of the hydrolyzate.

"Static mixing elements" are understood to be  
20 undulating crossmembers or strips of metal or plastic which intersect or which are interlocked in one another and which divide the space defined by the tube into a plurality of intersecting passages progressing along the axis of the tube. Elements of the type in question are  
25 marketed by Sulzer A.G. of CH-8401 Winterthur, for example under the trade-marks SMV, SMX or SMXL.

Finally, it is important to appreciate that the tubes provided with static mixing elements are systematically equipped with a double jacket even when this  
30 is not specifically mentioned.

The process according to the invention may be carried out using any starting material rich in proteins as the protein substrate, such as flours or semolina of oil seeds or oil seed cakes, food-quality yeasts or  
35 bacteria, minced animal or fish flesh or milk or milk derivatives,

for example in the form of particles in aqueous suspension or aqueous suspensions.

5 The protein substrate is preferably a whey substrate containing whey proteins, more particularly a sweet whey from cheese production or an acidic whey from casein production either as such or in demineralized or lactose-free, liquid or reconstituted form.

10 In another preferred embodiment, the enzyme is selected from the group consisting of trypsin, chymotrypsin, pancreatin, bacterial proteases, fungal proteases and mixtures thereof.

The proteolytic enzyme and the substrate may be mixed in a quantity of enzyme having an activity of 0.1 to 12 Anson units (AU) per 100 g substrate dry matter.

15 The first hydrolysis step is preferably carried out over a period of 10 to 60 minutes at a pH and a temperature adjusted to values favourable to the activity of the enzyme while the second hydrolysis step is preferably carried out over a period of 1 to 8 h at a temperature equal to or above, particularly 0 to 10°C above, the temperature of the first step.

20 Intermediate or complementary steps may be included, more particularly a preliminary mixing step preferably carried out in a tube equipped with static mixing elements; a thermal denaturing step before, in the middle of or after the first hydrolysis step, particularly using a heat exchanger or a tube equipped with static mixing elements; one or more enzyme inactivation steps, particularly after the second hydrolysis step, more especially using a heat exchanger and/or a steam injector and/or a tube equipped with static mixing elements; and/or a cooling step carried out in particular after a denaturing step, more particularly using a heat exchanger or preferably a tube equipped with static mixing elements for example.

35

The enzyme may be inactivated in one or preferably two steps, a first step corresponding more precisely to autodigestion of the enzyme and a second step corresponding more precisely to sterilization.

5           The first hydrolysis step in a tank may also be divided into at least two parts carried out in at least two tanks connected in series. Similarly, the second hydrolysis step carried out in a tube may be divided into at least two parts carried out in at least two  
10 tubes connected in series. In the latter case, a pH adjustment and/or an addition of enzyme may be carried out between two successive tubes.

          For the pH adjustment(s), it is preferred to use a suitable reactant which may either be alkaline, such  
15 as KOH, NaOH or  $\text{Ca}(\text{OH})_2$ , or acidic, such as HCl or  $\text{HPO}_4$ , for example.

          In one preferred embodiment of the process according to the invention, the enzyme is a bacterial alkaline protease, more particularly that produced by  
20 *Bacillus licheniformis* and marketed by the Novo company under the trade-mark of "Alcalase", more particularly "Alcalase 0.6 L" or "Alcalase 2.4 L" for example.

          It has been found that, with this preferred embodiment, it was possible to obtain a hydrolysate  
25 having a particularly high NPN and particularly reduced allergenicity.

          To this end, the first hydrolysis step is carried out at a pH value of 7.0 to 10.0 and at 50 to 80°C and preferably at 63 to 73°C while the second hydrolysis  
30 step is carried out at a pH value of 6.5 to 8.0 and at 55 to 80°C and preferably at 65 to 73°C. A thermal denaturing step may be carried out either after the first hydrolysis step or between the two hydrolysis steps over a period of 30 s to 10 mins. and preferably  
35 over a period of 4 to 6 mins. at a temperature of 80 to

120°C and preferably at a temperature of 85 to 95°C. The enzyme may then be inactivated by an autodigestion step carried out over a period of 10 s to 20 mins. and preferably over a period of 2 to 8 mins. at 70 to 110°C and preferably at 85 to 90°C, followed by a sterilization step carried out over a period 5 s to 5 mins. and preferably over a period of 30 s to 2 mins. at 110 to 150°C and preferably at 120 to 130°C.

In another preferred embodiment of the process according to the invention, the enzyme used is a combination of, on the one hand, a bacterial alkaline protease, more particularly that produced by *Bacillus licheniformis* and marketed by the Novo company under the name of "Alcalase", more particularly "Alcalase 0.6 L" or "Alcalase 2.4 L", and on the other hand a pancreatic enzyme, more particularly trypsin for example.

In this other preferred embodiment, two substrates, more particularly two whey substrates, may each be separately subjected to a separate hydrolysis with one of these two enzymes by the process according to the invention up to a common step, preferably up to a common sterilization step following two separate autodigestion steps of the two different enzymes. The same substrate may also be successively subjected to the action of one and then the other of these two enzymes. This is because it has been found that a hydrolysis product of whey proteins, for example, obtained by this combination can show particularly good stability in storage.

To carry out the process according to the invention with a pancreatic enzyme, particularly trypsin for example, above all in the above-mentioned combination, the pH and temperature conditions described in EP 322 589 may be used with advantage.

The apparatus for carrying out the process

according to the invention thus comprises a double-jacketed stirred hydrolysis tank which is connected upstream to a substrate metering unit and to an enzyme metering unit and downstream to at least one hydrolysis tube equipped with static mixing elements.

In this apparatus, the tube may be vertically arranged, its lower end being connected to the tank and its upper end opening into an outlet pipe. It may also be arranged horizontally or in any other position. In a preferred embodiment, it has a length of greater than 4 times its diameter.

In another preferred embodiment, the substrate and enzyme metering units each comprise a feed vessel connected to the hydrolysis tank by a volumetric pump.

The apparatus may also comprise a reactant metering unit comprising a feed vessel connected to the hydrolysis tank by a volumetric pump controlled by a pH meter.

The apparatus may also comprise several tanks connected in series instead of a single tank, more particularly two tanks of which one may be used as a prehydrolysis tank.

The apparatus may also comprise several hydrolysis tubes equipped with static mixing elements connected in series downstream of the tank by connecting pipes which may be connected upstream to the enzyme metering unit and to the reactant metering unit.

A tube equipped with static mixing elements may also be provided between the enzyme, substrate and/or reactant metering units and the tank or even between two successive tanks where the apparatus comprises several tanks.

The apparatus for carrying out the process according to the invention is described in more detail hereinafter with reference to the accompanying drawings

which illustrate three examples of embodiment and in which:

Figure 1 diagrammatically illustrates a first embodiment of the apparatus comprising a tank and a tube  
5 equipped with static mixing elements.

Figure 2 diagrammatically illustrates a second embodiment of the apparatus comprising a tank and several hydrolysis tubes equipped with static mixing elements.

10 Figure 3 diagrammatically illustrates a third embodiment of the apparatus comprising two tanks and several hydrolysis tubes equipped with static mixing elements.

Referring to Fig. 1, the present apparatus comprises a hydrolysis tank 1 with a double jacket 2 and a stirrer 3 driven by a motor 4. The tank is closed in fluid-tight manner by a cover 5 through pass various pipes and the shaft of the stirrer 3.  
15

The hydrolysis tank 1 is connected upstream by a pipe 6 to a substrate metering unit 7-11, by a pipe 12 to an enzyme metering unit 13-17 and by a pipe 18 to a reactant metering unit 19-24.  
20

The substrate metering unit comprises a substrate feed vessel 7 with a double jacket 8 and a stirrer 9 driven by a motor 10. The vessel 7 is connected to the hydrolysis tank 1 by the volumetric pump 11 connected to the pipe 6.  
25

The enzyme metering unit comprises an enzyme feed vessel 13 with a double jacket 14 and a stirrer 15 driven by a motor 16. The vessel 13 is connected to the hydrolysis tank 1 by the volumetric pump 17 connected to the pipe 12.  
30

The reactant metering unit comprises a reactant feed vessel 19 connected to the hydrolysis tank 1 by a volumetric pump 20 connected to the pipe 18. The  
35

volumetric pump 18 is controlled by a pH meter 21 of which the measuring electrode 24 dips into the tank 1 through the cover 5 and which is electrically connected (chain line 23) to an electronic device for controlling the pump 20 (not shown).

The hydrolysis tank 1 is connected downstream to a hydrolysis tube 25 with a double jacket 26 equipped with static mixing elements 27 consisting of metal or plastic crosspieces interlocked in one another. The tank 1 is connected to the tube 25 by a pipe to which is connected a three-way valve 29 designed to enable samples of hydrolyzate to be removed from the tank.

The tube 25 is vertically arranged, its lower end being connected to the tank 1 and its upper end opening into an outlet pipe 30.

The temperature of a fluid circulating in each of the double jackets is regulated by a device shown symbolically at 31 for the tank 1, at 32 for the vessel 7, at 33 for the vessel 13 and at 34 for the tube 25.

In Fig. 2, the elements of this second embodiment of the apparatus which correspond to the elements of the first embodiment shown in Fig. 1 are denoted by the same reference numerals.

In this second embodiment, the apparatus comprises several hydrolysis tubes 25, 35, 36 equipped with static mixing elements 27, 37, 38 and connected in series downstream of the tank 1 by connecting pipes 39, 40 connected upstream to the enzyme feed vessel 13 by pipes 41, 42 which rejoin the pipe 12 to which the volumetric pump 17 is connected.

The connecting pipes 39, 40 are also connected upstream to the reactant feed vessel 19 by pipes 43, 44 which rejoin the pipe 18 to which the volumetric pump 20 is connected.

In this second embodiment of the apparatus

according to the invention, a mixing tube 45 equipped with static mixing elements is again provided between the enzyme, substrate and reactant metering units and the tank 1.

5           The various enzyme, substrate and reactant feed vessels are connected to the tube 45 by pipes 46, 47 and 48 to which a volumetric pump 49 and the volumetric pumps 11 and 20 are respectively connected.

10           In Fig. 3, the elements of this third embodiment of the apparatus which correspond to the elements of the first two embodiments shown in Figs. 1 and 2 are again denoted by the same reference numerals.

15           In this third embodiment, the apparatus comprises several hydrolysis tubes 25, 35, 36, 50 equipped with static mixing elements and connected in series downstream of the hydrolysis tank 1. The hydrolysis tank is connected upstream to a second tank, in the present case a prehydrolysis tank 51, by a denaturing tube 52 equipped with static mixing elements.

20           In this third embodiment, it is the prehydrolysis tank 51 which is connected upstream to the substrate and reactant feed vessels 7 and 19 while the enzyme feed vessel 13 is connected downstream by the pipes 56, 12 and 41, respectively, to the prehydrolysis tank 51, the hydrolysis tank 1 and the pipe 39 connecting the hydrolysis tubes 25 and 35.

30           In this embodiment, inactivation tubes 53, 54 and a cooling tube 55 equipped with static mixing elements and connected in series downstream of the last hydrolysis tube 50 are again provided.

          The process according to the present invention is illustrated by the following Examples in which parts and percentages are by weight.

**Example 1**

The process according to the invention is carried out in an apparatus similar to that described with reference to Fig. 1, in which the hydrolysis tank has a  
5 volume of 30 l and the hydrolysis tube equipped with static mixing elements has a volume of 180 l for a height of 3 m.

The substrate used is a partly demineralized whey protein concentrate having a dry matter content of 20%  
10 and respective contents (in % based on dry matter) of approximately 23% proteins, 1.9% fats, 73% lactose and 1.3% ash.

Porcine trypsin having an activity of 3 AU/g is used as the enzyme in a quantity of 1 g enzyme to 100 g  
15 substrate dry matter, i.e. 3 AU to 100 g substrate dry matter.

2N KOH is used as reactant.

The tank is first filled with substrate and, after mixing in the enzyme, the discontinuous hydrolysis  
20 process is initiated at pH 7.3/60°C for 15 minutes, after which the hydrolyzed substrate has an NPN of 40%.

The process is then resumed continuously at such a rate that the mean residence time of the substrate in the tank is 30 minutes and the residence time of the  
25 hydrolyzate in the tube is 3 h. A temperature of 60°C and a pH of 7.3 are maintained in the tank. A temperature of 60°C is maintained in the tube, the pH being allowed to float so that it falls spontaneously from approximately 7.3 at the tube entrance to approximately  
30 6.9 at the tube exit.

The hydrolyzate has an NPN of 65% on leaving the tube.

If, for comparison, the same substrate is hydrolyzed discontinuously with the same enzyme in the same  
35 enzyme-to-substrate ratio for approximately 7 h at pH

7.3/60°C in a 200 litre tank, a hydrolyzate having an NPN of 60% is obtained.

**Example 2**

5           The procedure is the same as described in Example 1 except for the fact that, during three separate tests, the useful volume of the tank is varied so that the NPN obtained after the passage of the substrate through the tank is 15, 35 and 45%, respectively.

10           Hydrolyzates having respective NPN's of 59, 63 and 66% are thus obtained at the tube exit.

          For comparison, an NPN of 60% is obtained in approximately 7 h under the same conditions discontinuously in a tank, i.e. at pH 7.3/60°C with a substrate  
15           having a dry matter content of 20% and a quantity of enzyme having an activity of 3 AU per 100 g substrate dry matter.

          In other words, it is possible by the present process continuously to obtain an NPN higher than that  
20           which would be discontinuously obtained if the substrate had an NPN above 35% at the tube exit.

**Example 3**

          The procedure is the same as described in Example  
25           1 except that a pH value of 7.8 as opposed to 7.3 and a temperature of 55°C as opposed to 60°C are maintained in the tank.

          A hydrolyzate having an NPN of 70% is obtained at the tube exit.

30

**Example 4**

          The process according to the invention is carried out using an apparatus similar to that described with reference to Fig. 2.

35           A whey protein concentrate having a dry matter

content of 33%, including 7.5% proteins, is used as the substrate.

5 The enzyme used is a bacterial alkaline protease produced by *Bacillus licheniformis* and marketed by the Novo company under the name of "Alcalase 2.4 L" which has an activity of 2.4 AU/g. This enzyme is used in a total quantity of 4 to 8.6% based on protein, i.e. 2.2 to 4.7 AU per 100 g substrate dry matter.

2N KOH is used as the reactant.

10 After the process has been suitably initiated, it is resumed continuously. The throughput of substrate and the dimensions of the tube and the tank are determined in such a way that the residence times of the substrate or the hydrolyzate are, respectively, 5 to 10  
15 minutes in a preliminary mixing tube equipped with static mixing elements preceding the tank, 5 to 8 minutes in a thermal denaturing tube equipped with static mixing elements connected in series between the preliminary mixing tube and the tank, 25 to 40 minutes  
20 in the tank (first hydrolysis step), 15 to 25 minutes in a first tube A equipped with static mixing elements following the tank (tube A of the second hydrolysis step), 15 to 25 minutes in a second tube B equipped with static mixing elements (tube B of the second hydrolysis  
25 step), 0 to 100 minutes in a third tube C equipped with static mixing elements (tube C of the second hydrolysis step), 5 to 20 minutes in an inactivation tube equipped with static mixing elements connected in series to the remainder of the tube C and 5 to 15 minutes in a cooling  
30 tube equipped with static mixing elements.

The total quantity of enzyme is divided into 4 parts, namely a first part of 5 to 15% of the total mixed with the substrate in the preliminary mixing tube, a second part of 30 to 40% of the total mixed with the  
35 substrate in the tank, a third part of 20 to 30% of the

total mixed with the substrate in the tube A and a fourth part of 20 to 30% of the total mixed with the substrate in the tube B.

5 The pH value of the substrate is adjusted to 7.3 as far as the tube B from which the pH is left to float.

10 The temperature is adjusted to 75°C in the preliminary mixing tube, to 85°C in the thermal denaturing tube, to 70°C in the tank, to 71°C in the tube A and the tube B, to 80-105°C in the activation tube and to 2-8°C in the cooling tube.

The hydrolyzate thus produced is collected after the cooling tube.

#### Example 5

15 The present process is carried out using an apparatus of the type similar to that described with reference to Fig. 3.

20 A whey protein concentrate having a dry matter content of 28%, including 7% proteins, is used as the substrate.

Alcalase 2.4 L is used as the enzyme in a total quantity of 2 to 6% based on protein, i.e. 1.2 to 3.6 AU per 100 g substrate dry matter.

2N KOH is used as the reactant.

25 After the process has been suitably initiated, it is resumed continuously. The throughput of substrate and the dimensions of the tubes and the tanks are determined in such a way that the successive steps take place as follows.

30 In a preliminary mixing tube equipped with static mixing elements preceding a prehydrolysis tank, 33% of the total quantity of enzyme is mixed with the substrate at pH 8.7/10°C.

35 A first phase of the first hydrolysis step is carried out in the prehydrolysis tank for 15 minutes at

65°C.

In a thermal denaturing tube equipped with static mixing elements connected in series between the pre-hydrolysis tank and a hydrolysis tank, the temperature is increased to 92°C for 5 minutes followed by cooling to 65°C.

In the hydrolysis tank, the remaining 66% of the total quantity of enzyme is added and a second phase of the first hydrolysis step is carried out over a period of 45 minutes at pH 7.4/65°C.

In three tubes equipped with static mixing elements and connected in series downstream of the tank, the second hydrolysis step is carried out over a period of 195 minutes at 65°C, i.e. for 65 minutes in each tube. The pH is adjusted to 7.5 at the entrance of each tube and then floats.

In an inactivation tube equipped with static mixing elements connected in series after the three hydrolysis tubes, the enzyme is autodigested for 5 minutes at 87°C.

In a steam injection heating unit connected in series after the inactivation tube, the hydrolyzate is sterilized for 1 minute at 125°C.

The hydrolyzate is then collected after cooling.

#### **Example 6**

The present process is carried out using an apparatus similar to that described with reference to Fig. 1, in which the hydrolysis tank has a volume of 2.8 l and the hydrolysis tube equipped with static mixing elements has a volume of 11.6 l for a length of approximately 5 m.

A whey protein concentrate having a dry matter content of 33%, including 7.5% proteins, is used.

Alcalase 2.4 L is used as the enzyme in a total

quantity of 6.3% based on protein, i.e. 3.4 AU per 100 g substrate dry matter.

2N KOH is used as reactant.

5 The tank is first filled with substrate and, after mixing in the enzyme, the discontinuous hydrolysis process is initiated for 25 minutes at pH 7.3/70°C.

10 The process is then resumed continuously at such a rate that the total residence time of the hydrolyzate in the apparatus is 240 mins. (47 mins. in the tank and 193 mins. in the tube). A temperature of 70°C and a pH of 7.3 are maintained in the tank. A temperature of 70°C is maintained in the tube, the pH being allowed to float so that it falls spontaneously from approximately 7.3 at the tube entrance to approximately 6.72 at the tube exit.

15 Samples are taken for analysis at the tube exit at times of 0, 60, 120 and 180 minutes counting from 240 minutes after the start of the continuous process. These samples have the pH values and the amine nitrogen contents shown in Table I below where the corresponding quantity of KOH used to keep the pH at 7.3 in the tank is also shown.

**Table I**

| 25 | Time<br>(mins.) | pH   | Amine nitrogen<br>(%) | KOH<br>(g/h) |
|----|-----------------|------|-----------------------|--------------|
|    | 0               | 6.71 | 0.26                  | 124          |
|    | 60              | 6.72 | 0.25                  | 123          |
| 30 | 120             | 6.73 | 0.26                  | 125          |
|    | 180             | 6.71 | 0.26                  | 125          |

35 The quantities of KOH indicated in g/h correspond to an average consumption of 44.375 g per l of the tank for a residence time of 47 minutes.

It can be seen from Table I that the characteristics of the hydrolyzate hardly vary irrespective of the time the samples are taken for analysis from the tube exit. It is also possible to verify by zone electrophoresis in polyacrylamide gel (SDS-PAGE method) that the advantageous peptide profile of these samples, mostly small peptides, also remains remarkably constant.

For comparison, the same substrate is subjected to enzymatic hydrolysis with the same enzyme in the same enzyme-to-substrate ratio discontinuously for 47 minutes in a 2 litre tank at 70°C and at a pH kept at 7.3. After these first 47 minutes, the pH is left to float. Samples are taken for analysis after 47 minutes counting from the beginning of hydrolysis and then at various times up to and beyond 240 minutes. These samples have the pH values and amine nitrogen contents shown in Table II below.

**Table II**

|    | Time<br>(mins.) | pH   | Amine nitrogen<br>(%) |
|----|-----------------|------|-----------------------|
|    | 47              | 7.30 | 0.21                  |
| 25 | 67              | 7.0  | 0.22                  |
|    | 140             | 6.77 | 0.24                  |
|    | 197             | 6.75 | 0.27                  |
|    | 240             | 6.72 | 0.26                  |
|    | 300             | 6.68 |                       |
| 30 | 360             | 6.65 |                       |

The quantity of KOH used to keep the pH at 7.3 during the first 47 minutes is 45.5 g per litre of the tank.

It can be seen from Table II that the charac-

teristics of the hydrolyzate obtained discontinuously in a tank vary rapidly, again after the time of 240 minutes corresponding to the continuous residence time in the apparatus used in Example 6.

5           This demonstrates one of the advantages of the process according to the invention in which there is no danger of development of the product comparable with that occurring during the time required to empty the tank in a discontinuous process.

10

#### Example 7

          The present process is carried out using an apparatus similar to that described with reference to Fig. 1, in which the hydrolysis tank has a volume of 5  
15   1 and the hydrolysis tube equipped with static mixing elements has a volume of 9.6 l for a length of approximately 5 m.

          A whey protein concentrate having a dry matter content of 33%, including 7.5% proteins, is used as the  
20   substrate.

          Alcalase 2.4 L is used as the enzyme in a total quantity of 8% based on protein, i.e. 4.4 AU per 100 g substrate dry matter. Of these 8%, 2% are used in the tank and 6% are added at the tube entrance.

25           2N KOH is used as reactant.

          In two separate tests, the tank is first filled with substrate and, after mixing in the enzyme, the discontinuous hydrolysis process is started at pH 7.3 and at two different temperatures of 72.5°C and 74°C for  
30   40 minutes.

          Each process is then resumed continuously at such a rate that the total residence time of the hydrolyzate in the apparatus is 116 minutes (40 minutes in the tank and 76 minutes in the tube). Respective temperatures of  
35   72.5°C and 74°C and a pH of 7.3 are maintained in the

tank for each of the two tests. A temperature of 72°C is maintained in the tube, the pH being allowed to float.

5 The hydrolyzates thus obtained corresponding to the temperatures of 72.5°C and 74°C in the tank have respective NPN's of 97.2% and 91.4% and necessitated the use of respective quantities of 205 g/h and 198 g/h KOH to keep the pH at 7.3 in the tank. In addition, zone electrophoresis in polyacrylamide gel (SDS-PAGE method)  
10 shows that they have a relatively narrow peptide profile.

For comparison, the same substrate is subjected to enzymatic hydrolysis with Alcalase 2.4 L in a total quantity of 4% based on protein, i.e. 2.2 AU per 100 g  
15 substrate dry matter, continuously for 200 minutes in a 5 liter tank at 70°C and at respective pH values of 6.4, 6.8, 7.3 and 7.8 in four separate tests.

The hydrolyzates thus obtained have NPN's of 80 to 83% and necessitated the use of respective quantities  
20 of KOH (in g/h) of 33.4, 50.1, 60.2 and 77.2 to keep their pH values at 6.4, 6.8, 7.3 and 7.8. In addition, they have respective amine nitrogen contents of 0.17, 0.20, 0.21 and 0.22%. The SDS-PAGE test shows that they have a relative broad peptide profile.

25 This demonstrates another advantage of the process according to the invention insofar as it is possible to obtain a product having a high degree of hydrolysis and a relatively narrow peptide profile by comparison with a product obtained by continuous enzymatic hydrolysis in a tank which has a lower degree of  
30 hydrolysis and a relatively broad peptide profile.

#### Example 8

The present process is carried out using an  
35 apparatus similar to that described with reference to

Fig. 1, in which the hydrolysis tank has a volume of 2.8 l and the hydrolysis tube equipped with static mixing elements has a volume of 11.6 l for a length of approximately 5 m.

5           A whey protein concentrate having a dry matter content of 33%, including 7.5% proteins, is used as the substrate.

          Alcalase 2.4 L is used as the enzyme in a total quantity of 7% based on protein, i.e. 3.8 AU per 100 g  
10       substrate dry matter. Of these 7%, 2% are used in the tank and 5% are added at the tube entrance.

          2N KOH is used as the reactant.

          The tank is first filled with substrate and, after mixing in the enzyme, the discontinuous hydrolysis  
15       process is started at pH 7.8/70°C for 25 minutes.

          The process is then resumed continuously at such a rate that the residence time of the hydrolyzate is 45 minutes in the tank and 170 minutes in the hydrolysis tube, i.e. a total of 215 minutes. A temperature of  
20       70°C and a pH of 7.8 are maintained in the tank. A temperature of 70°C is maintained in the tube, the pH being allowed to float so that it falls spontaneously from approximately 7.8 at the tube entrance to approximately 6.67 at the tube exit.

25           In an inactivation tube equipped with static mixing elements and connected in series with the exit of the hydrolysis tube, the hydrolyzate is inactivated for 18 minutes at 90°C. In a cooling tube equipped with static mixing elements connected in series downstream of  
30       the inactivation tube, the hydrolyzate is cooled to ambient temperature.

          At the exit of the cooling tube, samples are taken for analysis at times of 0, 60, 120, 180 and 240 minutes counted at the exit of the hydrolysis tube from  
35       215 minutes after the start of the continuous process.

These samples have the pH values, amine nitrogen contents, lysine blockages and NPN's shown in Table III below where the corresponding quantity of KOH used to keep the pH at 7.8 in the tank is also shown.

5

**Table III**

|    | Time    | pH   | Amine<br>nitrogen | KOH   | Lysine<br>blockage | NPN |
|----|---------|------|-------------------|-------|--------------------|-----|
|    | (mins.) |      | (%)               | (g/h) | (%)                | (%) |
|    | 0       | 6.67 | 0.26              | 125   | 16.3               | 95  |
|    | 60      | 6.68 | 0.25              | 124   | 16.2               | 96  |
|    | 120     | 6.67 | 0.26              | 128   | 16.3               | 94  |
| 15 | 180     | 6.67 | 0.27              | 124   | 16.2               | 95  |
|    | 240     | 6.67 | 0.26              | 127   | 16.1               | 96  |

It can be seen from Table III in the same way as from Table I of Example 6 that the characteristics of the hydrolyzate hardly vary irrespective of the time at which the samples are taken for analysis at the exit of the hydrolysis tube.

The peptide profile and the hypoallergenic properties of the product obtained under the conditions of the present Example are also examined by subjecting it to the HPLC, ELISA and serotonin-<sup>3</sup>H tests of which the results are set out in Tables V, VI and VII below.

For comparison, 160 kg of the same substrate are subjected to discontinuous enzymatic hydrolysis with the same enzyme in a total quantity of 7% based on protein in a tank at 70°C. Of these 7% of enzyme, 2% are used for a first hydrolysis phase for 45 minutes at pH 7.8, after which the pH is left to float. After 60 minutes, the remaining 5% enzyme are added and hydrolysis is continued at 70°C and at a floating pH up to and beyond

215 minutes.

20 kg hydrolyzate are removed after 120 minutes counting from the beginning of hydrolysis. Further quantities of 20 kg are taken after 150, 180, 200, 250, 300 and 360 mins. A sample is taken for analysis after 215 minutes' hydrolysis.

The hydrolyzates corresponding to the various removals and samples are immediately inactivated (for 18 minutes at 90°C in a heat exchanger) and then cooled to ambient temperature (in a heat exchanger) and analyzed. They have the pH values, amine nitrogen contents (% based on powder containing 97% dry matter), lysine blockages and NPN's shown in Table IV below.

**Table IV**

| Time<br>(mins.) | pH   | Amine<br>nitrogen<br>(%, powder) | Lysine<br>blockage<br>(%) | NPN<br>(%) |
|-----------------|------|----------------------------------|---------------------------|------------|
| 60              | 7.56 |                                  |                           |            |
| 120             | 6.97 | 0.60                             | 15.3                      |            |
| 150             | 6.87 | 0.63                             | 17.2                      | 90         |
| 180             | 6.82 | 0.66                             | 18.2                      | 94         |
| 200             | 6.80 | 0.68                             | 18.3                      | 95         |
| 215             | 6.80 | 0.68                             | 18.9                      | 95         |
| 250             | 6.79 | 0.69                             | 19.4                      | 96         |
| 300             | 6.77 | 0.71                             | 19.5                      | 96         |
| 360             | 6.76 | 0.74                             | 19.6                      | 97         |

30

It can be seen from Table IV in the same way as from Table II in Example 6 that the characteristics of the hydrolyzate obtained discontinuously in a tank vary rapidly again after the 215 minutes corresponding to the continuous residence time in the apparatus used in

35

## Example 8.

This confirms one of the advantages of the present process in which there is no danger of development of the product comparable to that which occurs during the time required to empty the tank in a discontinuous process.

The peptide profile and the hypoallergenic properties of the product obtained under the conditions of the above Comparison Example are also examined after 215 minutes by subjecting the product to the HPLC, ELISA and serotonin-<sup>3</sup>H tests of which the results are set out in Tables V, VI and VII below.

Analogous tests are carried out on the product obtained continuously in a tank under the conditions corresponding to pH 7.3 presented for comparison with Example 7, the results also being set out in Tables V, VI and VII below.

**Table V                      Peptide profile (HPLC test)**

|    |                    |                                                                                                    |      |       |         |     |
|----|--------------------|----------------------------------------------------------------------------------------------------|------|-------|---------|-----|
| 20 | Product<br>acc. to | Percentage of peptides in the ranges<br>within the molecular weight limits<br>expressed in kDalton |      |       |         |     |
| 25 |                    | >14                                                                                                | 14-6 | 6-3.5 | 3.5-1.0 | < 1 |
|    | Example 8          | 5                                                                                                  | 7    | 9     | 30      | 49  |
|    | Comparison         | 4                                                                                                  | 7    | 10    | 31      | 48  |
| 30 | (disc. tank)       |                                                                                                    |      |       |         |     |
|    | Comparison         | 21                                                                                                 | 13   | 9     | 24      | 33  |
|    | (cont. tank)       |                                                                                                    |      |       |         |     |

The test results set out in Table V clearly illustrate the fact that a hydrolyzate continuously

obtained by the process according to the present invention can have a peptide profile at least as narrow and centred on the small peptides as a hydrolyzate obtained for comparison in a discontinuous tank whereas a hydrolyzate obtained for comparison in a continuous tank has a much broader peptide profile displaced towards the large peptides.

**Table VI ELISA inhibition test**

10

Product                      Residual antigenicity expressed in  
acc. to                      ug antigen per g protein

15

|                               | BLG | BSA    | CAS |
|-------------------------------|-----|--------|-----|
| Example 8                     | 53  | 20     | 150 |
| Comparison<br>(disc. tank)    | 41  | 7      | 141 |
| 20 Comparison<br>(cont. tank) | 111 | > 1000 | 319 |

**Table VII Serotonin-<sup>3</sup>H relaxation test**

25

Product                      Residual antigenicity expressed in  
acc. to                      ug of BLG equivalent for the relaxation  
per g protein equivalent

30

|                            |    |
|----------------------------|----|
| Example 8                  | 20 |
| Comparison<br>(disc. tank) | 5  |
| Comparison<br>(cont. tank) | 50 |

The test results set out in Tables VI and VII illustrate the fact that a hydrolyzate obtained by the process according to the present invention can be at least as hypoallergenic as a hydrolyzate obtained for comparison in a discontinuous tank whereas a hydrolyzate obtained for comparison in a continuous tank is not hypoallergenic.

#### Example 9

The present process is carried out in the same way as described in Example 7 under conditions corresponding to a temperature of  $72.5^{\circ}\text{C}$  in the tank. The tube is divided into nine segments. Samples are taken between two successive segments as the continuous hydrolysis progresses after the discontinuous initiation phase. Samples are taken at the tube exit at the same intervals when the duration of the continuous hydrolysis reaches the time corresponding to the residence time of the product in the tube.

The amine nitrogen content of the samples is determined. Each of these contents is divided by the equilibrium content towards which the hydrolyzate tends. On a system of coordinates, the quotients obtained are plotted as ordinates while the quotients of the sampling times divided by the residence time of the hydrolyzate in the apparatus are plotted on the abscissa.

A sigmoid curve is obtained, crossing from the abscissa 0.8, intersecting the vertical of the abscissa 1.0 at two thirds of its maximum value and reaching its maximum value, in other words touching the horizontal of the ordinate 1.0, at the vertical of the abscissa 1.2.

For comparison, the test is carried out in the same way except for the fact that the tube used is empty and, in addition, has the same dimensions as the tube equipped with static mixing elements.

Samples are taken under the same conditions, the same quotients are established and the corresponding curve is drawn in the same way.

5 A sigmoid curve is obtained, crossing from the abscissa 0.6, intersecting the vertical of the abscissa 1.0 at half its maximum value and only reaching its maximum value, i.e. 1.0, beyond the vertical of the abscissa 1.8.

10 This demonstrates another two advantages of the process according to the invention, namely on the one hand the rapidity with which steady-state conditions can be established and, on the other hand, the homogeneity of the hydrolyzate on leaving the apparatus.

## C L A I M S

1. A process for the enzymatic hydrolysis of proteins, in which a protein substrate is subjected to hydrolysis with a proteolytic enzyme comprising as a first step carrying out the enzymatic hydrolysis in a stirred tank and as a second step carrying out the enzymatic hydrolysis in a tube.
2. A continuous enzymatic hydrolysis process as claimed in claim 1, in which the second hydrolysis step is carried out in a tube equipped with static mixing elements.
3. A process as claimed in claim 1, in which the substrate is a starting material rich in proteins.
4. A process as claimed in claim 3, in which the starting material rich in proteins comprises flours or semolina of oil seeds or oil seed cakes, food-quality yeasts or bacteria, minced animal or fish flesh or milks or milk derivatives in the form of particles in aqueous suspension or an aqueous suspension.
5. A process as claimed in claim 1, in which the protein substrate is a whey substrate containing whey proteins.
6. A process as claimed in claim 5, in which the whey substrate containing whey proteins comprises a sweet whey from cheese production or an acidic whey from casein production in its normal form or in demineralized or lactose-free, liquid or reconstituted form.
7. A process as claimed in claim 1, in which the proteolytic enzyme is selected from the group consisting of trypsin, chymotrypsin, pancreatin, bacterial proteases,

fungus proteases and mixtures thereof.

8. A process as claimed in claim 1, in which the proteolytic enzyme and the substrate are mixed in such proportions that the enzyme activity is 0.1 to 12 AU per 100 g substrate dry matter.

9. A process as claimed in claim 1, in which the first step is carried out for 10 to 60 minutes at a pH and temperature adjusted to values favourable to the activity of the enzyme and the second step is carried out for 1 to 8 h at a temperature adjusted to a value equal to or above the temperature of the first step.

10. The process of claim 9, wherein the second step's temperature is 0 to 10°C above the first step's temperature.

11. An apparatus for carrying out the process claimed in claim 1, comprising a stirred hydrolysis tank connected upstream to a substrate metering unit and an enzyme metering unit and connected downstream to at least one hydrolysis tube.

12. An apparatus as claimed in claim 11, in which the hydrolysis tube is equipped with static mixing elements.

13. An apparatus as claimed in claim 12, in which the tube is vertically arranged, its lower end being connected to the tank and its upper end opening into an outlet pipe.

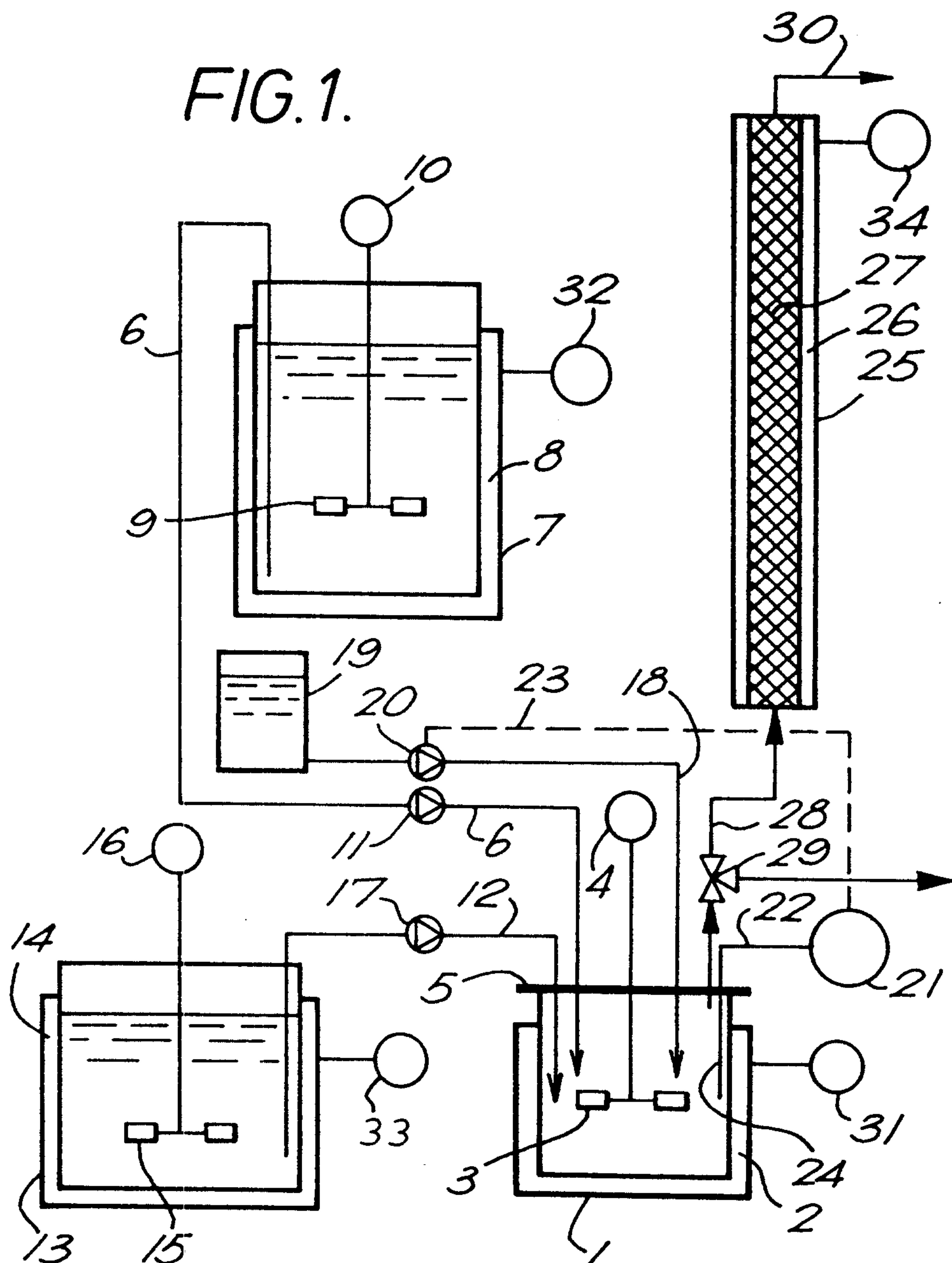
14. An apparatus as claimed in claim 12, in which the substrate and enzyme metering units each comprise a feed vessel connected to the hydrolysis tank.

15. An apparatus as claimed in claim 14, additionally comprising a reactant metering unit with a feed vessel

connected to the hydrolysis tank by a volumetric pump controlled by a pH meter connected to the tank.

16. An apparatus as claimed in claim 15, comprising several hydrolysis tubes equipped with static mixing elements and connected in series downstream of the tank by connecting pipes connected upstream to the enzyme metering unit and to the reactant metering unit.

FIG. 1.



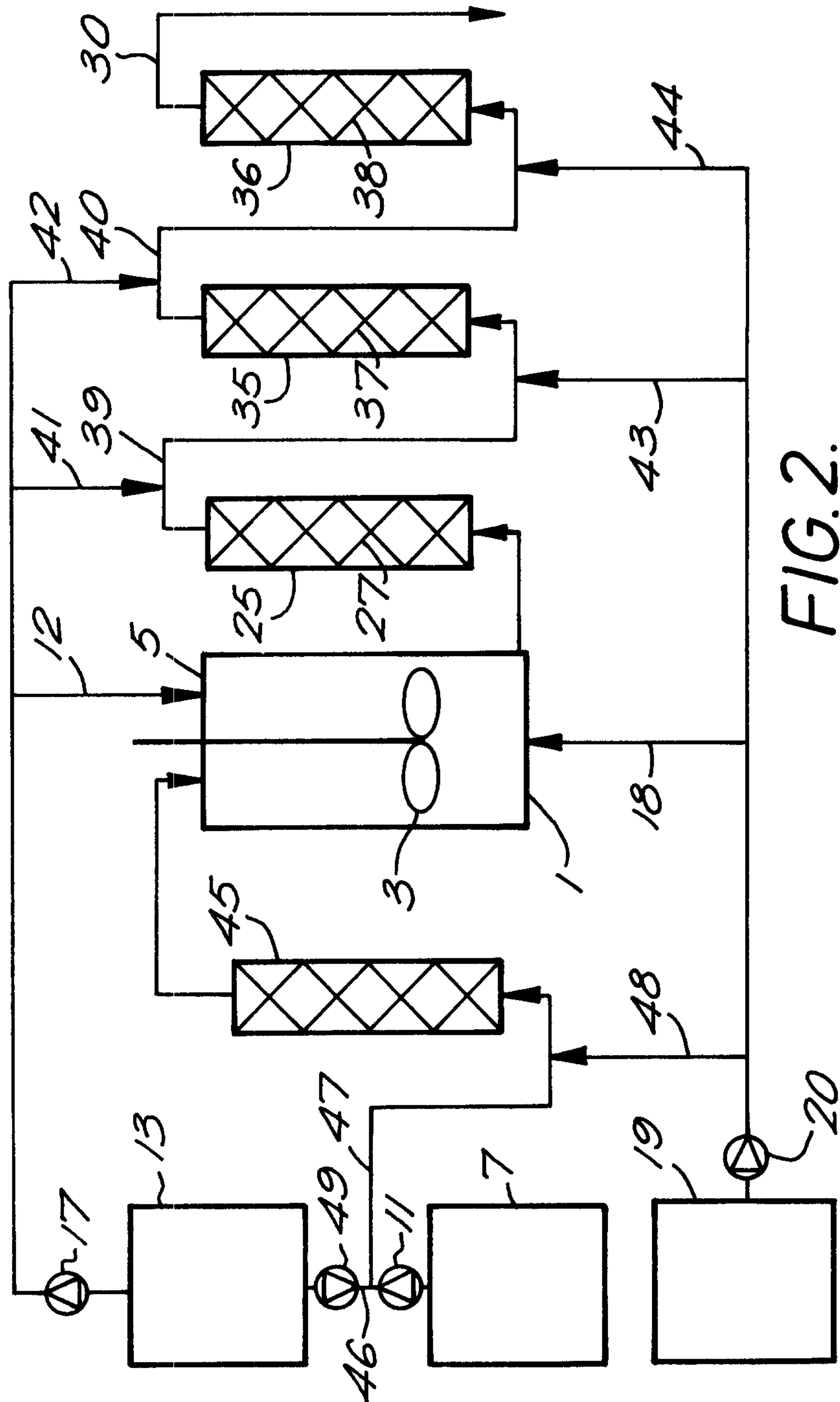


FIG. 2.

FIG. 3.

