



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b>  <b>C12N 9/00, 9/54</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 94/01537</b>  <b>(43) International Publication Date:</b> 20 January 1994 (20.01.94)
<b>(21) International Application Number:</b> PCT/DK93/00229 <b>(22) International Filing Date:</b> 6 July 1993 (06.07.93)  <b>(30) Priority data:</b> 0910/92                      10 July 1992 (10.07.92)                      DK  <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only) :</b> NIELSEN, Niels-Viktor [DK/DK]; Rye Gade 5, DK-4060 Kr. Såby (DK).  <b>(74) Common Representative:</b> NOVO NORDISK A/S; Novo Allé, DK-2880 Bagsværd (DK).		<b>(81) Designated States:</b> BR, FI, JP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHOD FOR PURIFICATION OF AN AQUEOUS ENZYME SOLUTION  <b>(57) Abstract</b>  The method comprises simultaneous addition of acid and aluminate in controlled amounts, optionally in the presence of a water miscible solvent, followed by separation of the supernatant from the precipitate. Hereby impurities are more effectively separated from the enzyme which remains in the supernatant.		

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## METHOD FOR PURIFICATION OF AN AQUEOUS ENZYME SOLUTION

In order to purify or concentrate an aqueous enzyme solution, especially an enzymatic fermentation broth, different purification methods are known in the art. Examples are salting-out and treatment with activated carbon. However, the selectivity, with which these purification methods separate enzymes from carbohydrates, non enzymatic proteins, colored compounds, and other impurities is open to improvement.

Thus, the purpose of the invention is the provision of a method for purification of an aqueous enzyme solution by means of which the enzymes can be effectively separated from carbohydrates, non enzymatic proteins, colored compounds, and other impurities. Usual sources of aqueous enzyme solutions are fermentation broths or filtered fermentation broths.

The method for purification of an aqueous enzyme solution according to the invention is characterized by the fact that a soluble aluminate is added to the aqueous enzyme solution, and that simultaneously an acid is added in such amounts that the pH-value of the solution is maintained between 4 and 10 and that the pH-value differs at least 1 pH unit from the isoelectric point of the enzyme to be purified, whereby the soluble aluminate is added to the solution in an amount between a maximum amount, which will precipitate no more than a negligible amount of enzyme and a minimal amount, which will leave no more than a negligible amount of impurities, mainly carbohydrates, in solution, and that the supernatant subsequently is separated from the precipitate, which is discarded.

It has been found that the precipitation process proceeds in the most efficient manner at lower pH-values. However, most enzymes exhibit low stability at low pH-values. Thus, a compromise pH-value with a reasonably efficient process and a reasonably good enzyme stability has to be chosen.

According to the invention it surprisingly has been found that the "window", i.e. the interval of added aluminate, in which practically no enzymes are precipitated and practically all impurities are precipitated, is larger than for other

precipitating agents, if the precipitation is carried out by means of the method according to the invention.

It is to be understood that the addition of the soluble aluminate to the pH-adjusted enzyme solution with subsequent precipitation of aluminum hydroxide will change the pH, but that according to the invention the pH-value will be readjusted with formic acid, if the changed pH-value should not fulfil the above indicated conditions, i.e. if the changed pH-value should not lie in the interval between 4 and 10, and if it should not differ at least 1 pH unit from the isoelectric point of the enzyme to be purified.

10 In this specification with claims "a soluble aluminate" means an aluminate, the solubility in aqueous medium of which is above 100 g/l of solution at a temperature of 25°C. An example is sodium aluminate.

The maximum amount of aluminate, which will precipitate no more than a negligible amount of enzyme is dependent upon the kind and the concentration of the enzyme, and it will normally be around 5% w/w aluminate in regard to the solution.

The minimal amount of aluminate, which will precipitate no more than a negligible amount of impurities, mainly carbohydrates, is dependent upon the kind and concentration of the impurities, and it will normally be around 0.5% w/w in regard to the solution.

A few enzymes will tend to precipitate to a certain degree already with addition of the soluble aluminate in an amount of 0.8% aluminate in regard to the solution, and such enzymes are not very well suited for this invention.

US patent no. 3,795,586 describes a purification method for enzymes, in which aluminum hydroxide and other insoluble compounds are precipitated in situ. However, in all examples, in which aluminum hydroxide is formed, aluminum sulphate was used, and it has been found that a much more effective separation of enzyme from impurities can be obtained by means of aluminate in combination with an acid, according to the invention.

30 In WO 91/09943, page 8, lines 11 - 13 it is indicated that  $\text{NaAlO}_2$  is added to a fermentation broth in an amount of 4 g of  $\text{NaAlO}_2$ /l of fermentation broth during the flocculation step. An addition of  $\text{NaAlO}_2$  in a concentration of that

size will not precipitate any dissolved material, but only flocculate already existing particles. Thus, this concentration of  $\text{NaAlO}_2$  is smaller than the minimum amount of aluminate indicated in Claim 1.

A preferred embodiment of the method according to the invention 5 comprises that the mixture comprising soluble aluminate, aqueous enzyme solution and acid also contains a water miscible solvent, preferably an alcohol. It has been found that the use of relatively high concentrations of aluminate (e.g. more than around 0.8%, calculated as Al) in some cases can generate substantial losses of enzymes during the initial flocculation, due to adsorption of the enzyme 10 on the aluminum containing flocs. This embodiment of the invention, however, can prevent these substantial losses. The alcohol is usually added prior to the flocculation, and this addition will not change the positive effect of the aluminate, and nor will it harm the separation of the supernatant from the precipitate.

A preferred embodiment of the method according to the invention 15 comprises that the alcohol is glycerol or a glycol. It has been found that glycerol or glycols are well suited alcohols for prevention of the above indicated substantial losses of enzyme.

A preferred embodiment of the method according to the invention comprises that the alcohol is present in the mixture in an amount of between 20 20 and 80% w/w. It has been found that the interval 20 - 80% w/w of alcohol is the optimal interval for the most efficient prevention of the substantial losses of enzymes.

A preferred embodiment of the method according to the invention comprises that the acid is formic acid. It has been found that formic acid provides 25 a more selective separation than other acids.

A preferred embodiment of the method according to the invention comprises that the pH-value of the solution is maintained between 5 and 8. This pH interval is optimal for most enzymes.

A preferred embodiment of the method according to the invention 30 comprises that the soluble aluminate is sodium aluminate. Sodium aluminate is the cheapest aluminate and is commercially available.

A preferred embodiment of the method according to the invention comprises that the maximum amount of aluminate is between 2 and 3% w/w of the solution, calculated as Al, and that the minimal amount is between 0.3 and 1% w/w of the solution, calculated as Al. These values for the maximum and 5 minimum additions of aluminate provide an efficient separation for most enzymes.

A preferred embodiment of the method according to the invention comprises that the supernatant is separated from the precipitate by centrifugation or filtration. These are the most efficient and simple methods of separation.

10 A preferred embodiment of the method according to the invention comprises that a flocculation agent is added after addition of the acid and the aluminate, but before the separation of the supernatant and the precipitate, and that the flocculation agent is a cationic and/or anionic flocculation agent. By use of these flocculation agents an even better separation is provided.

15 The method according to the invention will be illustrated by the following examples.

#### EXAMPLE 1

Savinase® is fermented according to US 3,723,250.

To 1 l of fermentation broth is added:

20 Water	1.5 l
Calcium chloride dihydrate	10 g
Sodium aluminate	40 -
Formic acid	approx. 40 -

pH is kept in the interval of 5.5 - 6.0 by control of the dosage of 25 formic acid

Rohafloc KF 785	approx.	300 mg
Superfloc A 130	-	150 -

The supernatant is separated from the precipitate by centrifugation.

Rohafloc KF 785 is a cationic flocculating agent from Röhm, and Superfloc A 130 is an anionic flocculating agent from American Cyanamid.

## EXAMPLE 2

5 Esperase<sup>®</sup> is fermented according to US 3,723,250.

After fermentation, the broth is pretreated by addition of 1 l tap water/l broth, 35 g of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ /l broth, and 10 g of  $\text{Al}_2(\text{SO}_4)_3$ /l broth. The pH is constantly adjusted to 8.0 with aqueous sodium hydroxide during the  $\text{Al}_2(\text{SO}_4)_3$  addition. The suspension is then flocculated by the addition of a cationic and an  
10 anionic flocculent: Superfloc C 521 (5 g/l broth) and Superfloc A 130 (300 mg/l broth).

Separation of the flocculated suspension is carried out by a centrifugation followed by a filtration in order to obtain a clear liquid. The filtrate is evaporated to a dry matter content corresponding to approx. 12% RI (refractive  
15 index) and heated to a temperature of approx. 37°C. At this temperature 250 g  $\text{Na}_2\text{SO}_4$ /l concentrate is added stepwise to the solution with rapid mixing. The pH is adjusted to 7.0 with sodium hydroxide and 20% acetic acid, and the suspension is mixed for 1/2 hour.

The precipitated enzyme is harvested on a filter press, and is  
20 redissolved in monopropylene glycol (2.5 l/kg filter cake) at 25°C. Undissolved material is removed by filtration, and the filter cake is flushed with a mixture of monopropylene glycol and water.

The Esperase<sup>®</sup> filtrate is diluted with water to a monopropylene glycol concentration of approx. 30%, the concentration of Esperase<sup>®</sup> being approx. 20  
25 g/l.

The monopropylene glycol concentrate is subsequently treated with sodium aluminate (5 g/l) at pH 5.9 (adjusted with formic acid) and filtered. The filter cake is flushed with a mixture of monopropylene glycol and water.

The yield of Esperase<sup>®</sup> in this aluminate reaction is above 95%, and  
30 the colour reduction is above 80%.

**CLAIMS**

1. Method for purification of an aqueous enzyme solution, characterized by the fact that a soluble aluminate is added to the aqueous enzyme solution, and that simultaneously an acid is added in such amounts that the pH-value of  
5 the solution is maintained between 4 and 10 and that the pH-value differs at least 1 pH unit from the isoelectric point of the enzyme to be purified, whereby the soluble aluminate is added to the solution in an amount between a maximum amount, which will precipitate no more than a negligible amount of enzyme and a minimal amount, which will leave no more than a negligible amount of impurities,  
10 mainly carbohydrates, in solution, and that the supernatant subsequently is separated from the precipitate, which is discarded.
2. Method according to Claim 1, characterized by the fact that the mixture comprising soluble aluminate, aqueous enzyme solution and acid also contains a water miscible solvent, preferably an alcohol.
- 15 3. Method according to Claim 2, characterized by the fact that the alcohol is glycerol or a glycol.
4. Method according to Claims 2 - 3, characterized by the fact that the alcohol is present in the mixture in an amount of between 20 and 80% w/w.
5. Method according to Claims 1 - 4, characterized by the fact that the  
20 acid is formic acid.
6. Method according to Claims 1 - 5, characterized by the fact that the pH-value of the solution is maintained between 5 and 8.



7. Method according to Claims 1 - 6, characterized by the fact that the soluble aluminate is sodium aluminate.
8. Method according to Claim 1 or 5 - 7, characterized by the fact that the maximum amount of aluminate is between 2 and 3% w/w of the solution, 5 calculated as Al, and that the minimal amount is between 0.3 and 1% w/w of the solution, calculated as Al.
9. Method according to Claims 1 - 8, characterized by the fact that the supernatant is separated from the precipitate by centrifugation or filtration.
10. Method according to Claims 1 - 9, characterized by the fact that a 10 flocculation agent is added after addition of the acid and the aluminate, but before the separation of the supernatant and the precipitate, and that the flocculation agent is a cationic and/or anionic flocculation agent.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 93/00229

## A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12N 9/00, C12N 9/54

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 3795586 (JACK ZIFFER), 5 March 1974 (05.03.74)  --	1-10
A	WO, A1, 9013632 (HENKEL KOMMANDITGESELLSCHAFT AUF AKTIEN), 15 November 1990 (15.11.90)  -- -----	1-10

 Further documents are listed in the continuation of Box C. See patent family annex.

## \* Special categories of cited documents:

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Date of the actual completion of the international search

5 October 1993

Date of mailing of the international search report

06 -10- 1993

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**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

26/08/93

International application No.  
PCT/DK 93/00229

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 3795586	05/03/74	CA-A- 947681 CA-A- 978489 DE-A- 2039752 FR-A- 2056864 NL-A- 7011845 US-A- 3700561	21/05/74 25/11/75 25/02/71 14/05/71 15/02/71 24/10/72
WO-A1- 9013632	15/11/90	DE-A- 3915277	15/11/90