Title: METHOD FOR REUSING A CARRIER FOR LIPASE IMMOBILIZATION

Abstract

A method for desorbing a hydrophobic protein, such as a lipase, from a carrier comprises treating a preparation comprising the hydrophobic protein immobilized by adsorption on a hydrophobic polymeric carrier with a desorption liquid comprising water, a water-miscible organic solvent and an alkali. The enzymatic activity of an immobilized lipase preparation which has wholly or partly lost its activity may be restored by such a desorption treatment followed by a readsorption treatment with fresh lipase. Immobilized lipase preparations regenerated in this manner are well suited as catalyst in, for example, the synthesis, hydrolysis or interesterification of esters.
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METHOD FOR REUSING A CARRIER FOR LIPASE IMMOBILIZATION

FIELD OF THE INVENTION

The present invention relates to a method for desorbing a hydrophobic protein, such as a lipase, from a carrier. The invention further relates to a method for regenerating or reactivating an immobilized lipase preparation by desorbing lipase protein from the carrier and adsorbing fresh lipase thereon, and to a process for the hydrolysis, synthesis or interesterification of an ester using an immobilized lipase.

BACKGROUND OF THE INVENTION

Enzymes have become a powerful catalytic tool in a wide variety of chemical processes. For many applications enzymes are preferably used in an immobilized state in order to easily separate the catalyst from the product stream. By way of example, an immobilized enzyme may be incorporated in a fixed bed reactor through which, or over which, a liquid reaction mixture is passed; alternatively, an immobilized enzyme may, for example, be employed as a solid (e.g. particulate) preparation which is added to a reaction mixture in a batch reactor (typically a stirred reactor) and subsequently - when the enzyme has performed its catalytic role - removed from the product mixture by filtration. It is now becoming feasible to exploit immobilized enzymes for catalysis on an industrial scale.

Although it is possible in many cases to reuse an immobilized enzyme catalyst several times before the catalytic activity of the immobilized enzyme(s) becomes lost, or at least reduced to an insufficient level, surprisingly little attention has been directed to ways of reusing the carrier material once the enzyme activity has become exhausted.

It is highly desirable, both from an economic and from an environmental viewpoint, to be able to recycle the carrier material, but it has generally been found very difficult to remove spent enzymes (i.e. enzymes that have wholly or partly lost their activity,
e.g. as a result of denaturing) from a carrier material to which they are adsorbed. One object of the present invention is to provide a simple process by which the carrier material may be reused and reloaded with fresh enzyme.

One group of industrially useful enzymes are the lipases (i.e. lipolytic enzymes), which are hydrophobic proteins that act on carboxylic acid esters, such as glyceride lipids, at the interface between an aqueous phase and an oily phase (e.g. a lipid phase). The immobilization of lipases, and the use of immobilized lipases in lipase-catalyzed reactions such as ester hydrolysis, ester synthesis and/or interesterification, are well known.

One approach to the immobilization of lipases is the adsorption thereof on a hydrophobic carrier. Thus, for example, EP-A-0 232 933 describes hydrolysis of fats using lipase immobilized by adsorption from aqueous solution on a microporous structure comprising a hydrophobic polymer such as polypropylene. This document mentions (page 6, lines 24-32) that the immobilized lipase may be used in a packed column, and that lipolytic activity of the column may be restored by a procedure wherein the column is first flushed with a solvent such as alcohol, then flushed with water to remove the solvent, further flushed with a broth of fresh lipase and finally flushed with water to wash away excess enzyme.

JP-A 51-15687 (Agency of Industrial Science and Technology) discloses a method wherein a water-miscible solvent (e.g. ethanol) is employed for the recovery of a lipase adsorbed on an insoluble carrier having hydrophobic residues bound to it. In order to improve the recovery of the lipase, it is preferred in some cases to elute with a mixture of a water-miscible solvent and an aqueous solution containing a buffer; the highest pH values used in this context appear to be below 9.

The present inventors have found that the above approach does not adequately remove spent lipase, and that the catalytic activity of the resulting "regenerated" immobilized lipase is unsatisfactory. The present invention provides provide a
"regeneration" process whereby very satisfactory lipolytic activity of the "regenerated" immobilized lipase is obtained.

STATEMENT OF THE INVENTION

It has surprisingly been found that hydrophobic proteins can be removed very effectively from certain polymeric carrier materials by treatment (e.g. flushing or elution) with a liquid comprising water, an alkali and a water-miscible solvent such as a lower alcohol. The method is very simple and works surprisingly well. It is usually very difficult to displace proteins such as enzymes that have been denaturated and lost activity, but with the methodology of the present invention it is possible to achieve a very high degree of displacement of enzyme protein from the carrier and to subsequently "regenerate" the immobilized lipase so as to obtain nearly the same maximal catalytic activity of the "regenerated" immobilized enzyme preparation as in the case in which the carrier material is used for the first time (i.e. in which a fresh, previously unused sample of carrier material is loaded with enzyme for the first time).

With the procedure of the present invention, it is thus possible, for example, to charge a column reactor with carrier material (i.e. support material or matrix) and then conduct all the various steps (such as immobilization of enzyme for the first time; performance of the enzyme-catalysed reaction in question by passage of the reaction mixture through the reactor column; displacement of spent enzyme; and regeneration of the immobilized enzyme preparation) in situ on the column reactor.

The very high percentage retention of enzymatic activity attainable in each regeneration step can significantly increase the useful lifetime of a given charge of carrier material in such a column reactor, and because of its simplicity the overall procedure requires considerably less manpower - and is therefore economically more attractive - than a procedure in which the maintaining of satisfactory enzymatic activity in the column reactor entails that the reactor be completely recharged with
new carrier material after at best a very few use/regeneration cycles, and/or that the carrier material be removed from the reactor for regeneration.

Accordingly, a first aspect of the invention provides a method for desorbing a hydrophobic protein from a carrier, wherein a preparation or composition comprising the hydrophobic protein adsorbed (i.e. immobilized by adsorption) on a hydrophobic polymeric carrier material is treated with a desorption liquid comprising water, a watermiscible organic solvent and an alkali.

A second aspect of the invention provides a method for desorbing a lipase from a carrier, wherein a preparation or composition comprising the lipase immobilized by adsorption on a hydrophobic polymeric carrier is treated with a desorption liquid comprising water, a water-miscible organic solvent and an alkali.

In a further aspect, the present invention provides a method for reactivating or regenerating an immobilized lipase preparation, comprising the steps of:

a) treating a preparation comprising lipase immobilized by adsorption on a hydrophobic polymeric carrier with a desorption liquid comprising water, a water-miscible organic solvent and an alkali, so as to desorb lipase from the carrier, and

b) adsorbing fresh lipase on the thus-treated carrier.

A still further aspect of the invention provides a process for hydrolyzing, synthesizing or interesterifying an ester, comprising the following steps (normally in the given relative order):

a) providing an immobilized lipase preparation comprising lipase immobilized by adsorption on a hydrophobic polymeric carrier,

b) contacting reactants with the immobilized lipase preparation, the reactants being: in the case of ester hydrolysis: the ester together with water,
in the case of ester synthesis: an acid together with an alcohol, or
in the case of interesterification: the ester together with an acid, an alcohol or a
second ester,

c) treating the immobilized lipase preparation with a desorption liquid comprising
water, a water-miscible organic solvent and an alkali, so as to desorb lipase from the
carrier,

d) immobilizing fresh lipase on the thus-treated carrier by adsorption of lipase
thereon, and
e) repeating step b).

10 In connection with the latter process of the invention, it will generally be possible and
advantageous to repeatedly regenerate a given sample of immobilized enzyme
preparation [in accordance with steps c) and d)] and reuse it in step b), i.e. to
repeat the sequence of steps c)-e) one or more times.

DETAILED DISCLOSURE OF THE INVENTION

15 Carrier
As already indicated, the carrier used in the invention is a hydrophobic polymer. The
hydrophobic polymer is very suitably an aliphatic olefinic polymer, particularly
polypropylene, or a homo- or co-polymer of monomers selected from divinyl-
benzene and C_{1-4} alkyl acrylates (including C_{1-4} alkyl methacrylates), particularly
methyl and butyl methacrylate. Very suitable hydrophobic polymeric carriers are
polymers and co-polymers which are uncharged, i.e. carriers lacking
groups/substituents which have - or which under appropriate conditions of, e.g., pH
may acquire - a positive or negative charge (examples of such charged carriers
would be polymers and co-polymers possessing ion-exchange properties, e.g. as
the result of the presence of quaternary ammonium groups or strongly acidic groups
such as sulfonic acid groups).
Preferred carriers will generally be porous (microporous or macroporous), and the carrier is preferably in the form of discrete particles which may be contacted with a liquid medium (e.g. reaction medium or desorption liquid) by stirring, or by allowing the liquid medium to flow through a packed column of such particles. Alternatively, the carrier may in the form of a "diaphragm" comprising a layer of immobilization carrier in fibrous form, e.g. a diaphragm constructed as described in EP 0 232 933 A1 (see, for example, page 4, lines 33-41, the paragraph bridging pages 6 and 7, as well as Fig. 3 and the associated description of Fig. 3 therein).

Hydrophobic protein

The hydrophobic protein may be any protein containing a hydrophobic region which is preferentially present at an oil/water interface. It may very suitably be an enzyme capable of hydrolyzing carboxylic ester bonds (e.g. at an oil/water interface), i.e. an enzyme (a "carboxylic ester hydrolase") classified under EC 3.1.1 in accordance with the authoritative "Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology" given in Enzyme Nomenclature, Academic Press Inc., 1992. Particularly suitable carboxylic ester hydrolases are lipases, more specifically triacylglycerol lipases (EC 3.1.1.3) capable of hydrolyzing ester linkages in triglycerides; triacylglycerol lipases may be positionally specific (often 1,3-specific) or positionally non-specific.

Examples of relevant lipases are microbial lipases derived from bacteria such as Pseudomonas (e.g. P. cepacia), from fungi such as Aspergillus or from yeasts such as Mucor (e.g. M. miehei), Humicola (e.g. H. lanuginosa) or Candida (e.g. C. antarctica). One preferred lipase is C. antarctica lipase B (described in WO 88/02775).

Immobilized lipase

The lipase is immobilized on the carrier by adsorption. The immobilization may be done by methods known in the art. Immobilization on a hydrophobic carrier such as polypropylene is described, e.g., in EP 0 232 933 A1 (AKZO N.V.). The hydrophobic
carrier may advantageously be pretreated by wetting with an alcohol such as ethanol, as described in the latter European patent application.

In a preferred embodiment, the immobilized lipase is in particulate form in a packed column, and it is preferable that the lipase-catalyzed reaction in question, the desorption of lipase from the carrier, and the regeneration procedure (i.e. adsorption of fresh lipase on the carrier) are all carried out in situ, i.e. while the carrier is packed in the column.

Desorption liquid
As already mentioned, the liquid used for desorption according to the invention comprises water, a water-miscible organic solvent and an alkali. The solvent is preferably a C_{1-4} alcohol, i.e. an alcohol selected from methanol, ethanol, 1- and 2-propanol and butanols, particularly ethanol or 2-propanol. The liquid preferably contains from 30 to 70% by weight (w/w) of water and from 30 to 70% w/w of the solvent.

The term "alkali" as employed in the present specification and claims is used to denote a substance (a base) which at moderate to high concentrations in aqueous solution gives rise to a strongly alkaline solution. Suitable alkalis may be selected from the hydroxides and carbonates of sodium and potassium, particularly the hydroxides thereof. The alkali should normally be present in the desorption liquid at a concentration of at least 0.005 equivalents/liter (i.e. at least 0.005 N). The alkali concentration will normally be in the range of 0.005-4 N, but more preferably in the range of 0.5-4 N. The desorption liquid should normally have a pH of at least 12 (i.e. 12 or above), and preferably at least 13 (i.e. 13 or above). A pH in the vicinity of 14 will often be appropriate.

The desorption treatment may be carried out at a temperature in the range of 5-85°C for a period of 1-36 hours, particularly 20-40°C for 1-4 hours.
Lipase-catalyzed reaction
The lipase-catalyzed reaction may be ester hydrolysis, ester synthesis or interesterification, and may be carried out in a conventional manner. Thus, the ester involved may be any carboxylic acid ester, e.g. a triglyceride or a sugar ester.

5 Lipase activity by the LU method
In this method, the lipase activity is measured at 30°C and pH 7.0 with Tributyrin (i.e. glyceryl tributyrate) as the substrate. One Lipase Unit (LU) is the amount of enzyme which liberates 1 μmol of titratable butyric acid per minute under these conditions. 1 KLU (Kilo Lipase Unit) is equal to 1000 LU.

10 Lipase activity by the EGEU method
In this method, the activity of an immobilized lipase preparation is measured by ester synthesis from ethyl glucoside and decanoic acid (1:2 weight ratio) in a small stirred reactor at 70°C to obtain less than 25% conversion. 1 EGEU (Ethyl Glucoside Ester Unit) corresponds to the production of 1 μmol of ester per minute per g of the immobilized lipase.

EXAMPLES

Example 1. Batch-wise desorption of lipase

*Candida antarctica* lipase B (see WO 88/02775) was immobilized by adsorption on particulate microporous polypropylene (Accurel™ EP 100; product of AKZO, Holland) at a loading of 92 KLU/g of the carrier. Batches of the resulting immobilized lipase preparation were agitated with various mixtures of water, alcohol and alkali, as detailed in the table below, and the extent of protein desorption was determined by measuring the optical density (OD) at 280 nm in the resulting liquid phase.

The experimental conditions and the OD values measured after 1, 1.5, 2, 2.5 and 4 hours, respectively, of treatment were as shown in the table below. The balance of
each liquid was water. Based on the amount of adsorbed lipase, it is estimated that full desorption would result in an OD of about 1.7.

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<td>2.1</td>
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§EP 0 232 933 A1

It is apparent that the lipase can be readily desorbed using a mixture of water, ethanol and alkali (NaOH) according to the invention. It is likewise apparent that the use of a desorption liquid containing water and ethanol but no alkali leads to very little desorption of lipase, whilst the use of ethanol alone (as described in EP 0 232 933 A1), which contains only a very small amount of water, in the absence of alkali results in essentially no desorption of lipase.

Some analogous experiments were carried out on the same type of immobilized lipase preparation to examine the protein-desorption effect of treatment with aqueous alkali alone (i.e. with a liquid containing no ethanol). The results obtained using a
M solution of NaOH in water alone revealed only a poor degree of protein desorption, thereby demonstrating the importance of the alcohol (water-miscible solvent) component in the desorption liquid for achieving satisfactory lipase desorption.

Example 2. Immobilized lipase on polypropylene in packed column

Preparation of immobilized lipase

Candida antarctica lipase B was immobilized on a polypropylene carrier (Accurel™ EP 100; particle size 200 - 800 μm) in a conventional manner, and the immobilized enzyme preparation was loaded into a column.

Ester synthesis

The column containing immobilized lipase was used for continuous ester synthesis in a conventional manner for several weeks, after which the lipase activity was essentially exhausted.

Desorption of lipase protein from the carrier

A 2.7 g sample of the spent immobilized lipase remaining after ester synthesis was placed in a column, and 200 ml of a desorption liquid in the form of a 1M solution of NaOH in 50% (w/w) aqueous ethanol was recirculated over the column bed at a flow rate of 1 ml/min for 2 hours at 25°C. The column was then washed with water until the effluent was of neutral pH.

The total protein content of the carrier was determined by conventional Kjeldahl analysis before and after the desorption + washing process. The results are given in the table below. Results are also given for the Accurel™ EP 100 carrier material before immobilization of lipase thereon (blank or baseline value) and for the immobilized lipase preparation before carrying out ester synthesis. The data indicate approximately 97% efficiency of protein removal from the carrier using the described desorption procedure.
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<th>Sample</th>
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<td>Carrier before use</td>
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<td>After immobilization</td>
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<td>After ester synthesis</td>
<td>9.8</td>
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<tr>
<td>After desorption</td>
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**Adsorption of fresh lipase on the carrier**

Fresh *C. antarctica* lipase B was adsorbed onto the carrier material resulting from the above-described desorption procedure. For this purpose, a volume of lipase solution (pH 6, 25°C) containing a total of 216,000 LU of lipase was circulated over the carrier material at a flow rate of 1 ml/min. The procedure was conducted overnight, and the resulting "regenerated" immobilized lipase preparation was then washed with water to remove excess lipase solution and finally with 96% ethanol to remove water.

The immobilization loading for the regenerated material was determined as 75,400 LU/g carrier (95% immobilization yield), essentially the same as when using the same immobilization conditions but a new sample of carrier. The lipase activity of the regenerated preparation was 694 EGEU, which is also essentially the same as obtained starting with a new sample of carrier.

Some analogous lipase re-adsorption experiments were carried out on a sample of immobilized *C. antarctica* lipase B on Accurel EP-100 carrier which had been subjected (see the last paragraph of Example 1, above) to a treatment with a desorption liquid consisting only of 1 M aqueous NaOH (i.e. containing no ethanol or other water-miscible organic solvent) in order to examine the extent of regeneration of lipase activity achievable. The results indicated a much lower lipase loading than that obtained with fresh carrier material.
It is thus apparent that the presence of the alcohol component in an alkaline desorption liquid (in accordance with the teaching herein) is important not only for achieving satisfactory desorption of enzyme protein from the carrier, but also for achieving a satisfactory degree of regeneration of enzyme activity in an immobilized enzyme preparation.

Example 3. Divinylbenzene/acrylate copolymer carrier in packed column

*C. antarctica* lipase B was immobilized on a macroporous copolymer (Lewatit® VPOC 1600, particle size 160-1000 µm; product of Bayer, Germany) prepared from divinylbenzene, methyl methacrylate and butyl methacrylate. The resulting immobilized lipase preparation was used for ester synthesis, lipase desorption and lipase absorption in the same manner as in Example 2.

The total protein content of the carrier before enzyme immobilization (blank or baseline value), after ester synthesis (i.e. before protein desorption), and after protein desorption was determined as before by conventional Kjeldahl analysis, and the results are given in the table below. The results indicate >98% desorption of protein by means of the desorption procedure in question.

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<th>Sample</th>
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<td>Before desorption</td>
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<td>After desorption</td>
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<td>Carrier before use (blank)</td>
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**Adsorption of fresh lipase on the carrier**

Fresh *C. antarctica* lipase B was adsorbed onto the carrier material resulting from the above-described desorption procedure. Lipase adsorption and subsequent washing with water and ethanol were performed in the same manner as in Example
2, except that a volume of lipase solution (pH 6, 25°C) containing a total of 212,000 LU of lipase was circulated over the carrier material.

The immobilization loading for the regenerated material was determined as 132,000 LU/g carrier, to be compared 140,000 LU/g carrier when using the same immobilization conditions but a new sample of carrier. The measured lipase activity of the regenerated preparation (determined by the EGEU method) was slightly higher than that of the immobilized lipase preparation obtained starting with a new sample of carrier.
CLAIMS

1. A method for desorbing a hydrophobic protein from a carrier, comprising: treating a preparation comprising the hydrophobic protein immobilized by adsorption on a carrier which is a hydrophobic polymer with a desorption liquid comprising water, a water-miscible organic solvent and an alkali.

2. A method according to claim 1, wherein said protein is a lipase.

3. A method for reactivating an immobilized lipase preparation, comprising the steps of:

   a) treating a preparation comprising lipase immobilized by adsorption on a carrier which is a hydrophobic polymer with a desorption liquid comprising water, a water-miscible organic solvent and an alkali so as to desorb lipase from the carrier, and

   b) adsorbing fresh lipase on the carrier.

4. A process for the hydrolysis, synthesis or interesterification of an ester, comprising the following steps:

   a) providing an immobilized lipase preparation comprising lipase immobilized by adsorption on a carrier which is a hydrophobic polymer,

   b) contacting reactants with the immobilized lipase preparation, said reactants being: in the case of ester hydrolysis: the ester and water, in the case of ester synthesis: an acid and an alcohol, or in the case of interesterification: the ester and an acid, an alcohol or a second ester,

   c) treating the immobilized lipase preparation with a desorption liquid comprising water, a water-miscible organic solvent and an alkali so as to desorb lipase from the carrier,
d) adsorbing fresh lipase on the carrier, and

e) repeating step b).

5. A process according to claim 4, wherein the sequence of steps c) - e) is repeated one or more times.

6. A process according to any one of claims 3-5, wherein the immobilized lipase preparation in step a) is provided as discrete particles in a packed column, and steps b) and, where relevant, c) and d) are performed *in situ* by passing the reagents or reactants in question through the column.

7. A process according to any one of the preceding claims, wherein the water-miscible organic solvent is a C_{1-4} alcohol.

8. A process according to claim 7, wherein the organic solvent is selected from the group consisting of ethanol and isopropyl alcohol.

9. A process according to any one of the preceding claims, wherein the desorption liquid comprises 30-70% by weight of water and 30-70% by weight of the water-miscible organic solvent.

10. A process according to any one of the preceding claims, wherein the alkali is selected from the group consisting of sodium hydroxide, sodium carbonate, potassium hydroxide and potassium carbonate.

11. A process according to any one of the preceding claims, wherein the desorption liquid contains 0.005-4 N of the alkali and/or has a pH above 12.

12. A process according to any one of the preceding claims, wherein the desorption liquid contains 0.5-4 N of the alkali and/or has a pH above 13.
13. A process according to any one of the preceding claims, wherein the carrier is a hydrophobic, aliphatic olefinic polymer.

14. A process according to any one of the preceding claims, wherein the carrier is polypropylene.

15. A process according to any one of the preceding claims, wherein the hydrophobic carrier is a hydrophobic homo- or co-polymer of monomers selected from the group consisting of divinylbenzene and C₄₋₅ alkyl acrylates.

16. A process according to any one of the preceding claims, wherein the hydrophobic carrier is a hydrophobic homo- or co-polymer of monomers selected from the group consisting of divinylbenzene, methyl methacrylate and n-butyl methacrylate.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 11/08, C12N 11/00 // C12M 1/40, C12P 7/64
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)

IPC6: 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)

BIOSIS, DERWENT BIOTECHNOLOGY ABSTRACT, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>US 4933284 A (C. D. LAPINS ET AL), 12 June 1990 (12.06.90), example 2, claim 1</td>
<td>1-16</td>
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<tr>
<td>X</td>
<td>EP 0232933 A1 (AKZO N.V.), 19 August 1987 (19.08.87), page 6, line 28 - line 32</td>
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<td>ANALYTICAL CHEMISTRY, Volume 46, No 7, June 1974, H. H. Weetall, &quot;Immobilized Enzymes&quot;, page 602 - page 615, see p 602, column 2, line 28 - column 3, line 4</td>
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Date of the actual completion of the international search: 12 Sept. 1995

Date of mailing of the international search report: 19 -09- 1995

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