

PATENT SPECIFICATION

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- (72) Inventors FRITZ HUPER, ERICH RAUENBUSCH
 GUNTER SCHMIDT-KASTNER
 BRUNO BOMER and HERBERT BARTL

(54) NEW WATER-INSOLUBLE PREPARATIONS OF PEPTIDE MATERIALS, THEIR PRODUCTION AND THEIR USE

(71) We, BAYER AKTIENGESELL-
 SCHAFT, a body corporate organised under
 the laws of Germany, of 509 Leverkusen
 Bayerwerk, Germany, do hereby declare the
 invention for which we pray that a patent
 may be granted to us and the method by
 which it is to be performed to be particularly
 described in and by the following state-
 ment:—

The invention relates to new water-insoluble
 preparations in which a polypeptide material
 is bound to a copolymer, to a process for the
 production of these new preparations, and to
 their use for carrying out enzyme-catalysable
 reactions. The invention constitutes a modi-
 fication of the invention in copending Appli-
 cation No. 15114/73 (Serial No. 1413381).

The covalent bonding of substances to in-
 soluble polymeric carriers has increasingly
 gained in importance in recent years. The
 binding of catalytically active compounds, for
 example enzymes, offers particular advantages
 since they can, in this form, easily be separ-
 ated off after completion of the reaction and
 be repeatedly re-used.

Copolymers of maleic anhydride and vinyl
 compounds have already repeatedly been pro-
 posed as carriers with suitable binding groups.
 However, copolymers of maleic anhydride
 with ethylene and monovinyl compounds be-
 come more or less water-soluble on reaction
 with aqueous enzyme solutions so that before
 or during the reaction an additional cross-
 linking agent, for example a diamine, is de-
 sirable added. Enzyme preparations obtained
 in this way are relatively difficult to filter
 and possess soluble constituents, and losses
 of bound enzyme result (compare E. Katchal-
 ski, *Biochemistry* 3, (1964), pages 1905—
 1919).

Further, copolymers of acrylamide and

maleic acid have been described, which are
 converted into the anhydride form by subse-
 quent heating. These products are relatively
 slightly crosslinked, swell very markedly in
 water and only possess moderate mechanical
 stability, which leads to abrasion losses when
 using these resins (compare German
 Offenlegungsschrift 1 908 290).

Furthermore, strongly crosslinked carrier
 polymers have been produced by copolymer-
 isation of maleic anhydride with divinyl ethers.
 Because of the alternating type of copoly-
 merisation of the monomers, these polymers
 contain a very high proportion of anhydride
 groups—in each case above 50% by weight
 of maleic anhydride, in the examples dis-
 closed—which is determined by the molecular
 weight of the vinyl ether monomer and is
 therefore only adaptable to the particular end
 use within relatively narrow limits (compare
 German Offenlegungsschrift (Published Speci-
 fication) 2,008,996).

We have therefore attempted to find new
 reaction products of proteins and peptides
 with new, strongly crosslinked, water-swell-
 able copolymers possessing a content of cyclic
 dicarboxylic acid anhydride groups capable of
 great variation, and a convenient process for
 their production. The new reaction products
 of proteins and peptides with the new copoly-
 mers should not have the disadvantages of
 the previously known protein preparations or
 should only have them to a lesser extent.

We now provide, according to this inven-
 tion, a water-insoluble preparation comprising
 a peptide material (as hereinafter defined)
 bound to a crosslinked copolymer comprising
 the following copolymerised residues (per-
 centages being by weight of the cross-linked
 polymer):

A: 0.1 to 30 per cent, preferably 2 to 20

- per cent, of at least one α,β - monoolefinically unsaturated dicarboxylic acid anhydride having 4 to 9 carbon atoms, preferably 4 to 5 carbon atoms;
- 5 B: 35 to 90 per cent, preferably 50 to 85 per cent, of at least one di- and/or polyacrylate or methacrylate of a diol and/or a polyol (as hereinafter defined); and
- 10 C: 5 to 60 per cent, preferably 10 to 50 per cent, of at least one hydrophilic monomer (as hereinafter defined);
- the copolymer having a bulk volume of 1.4 to 30, (preferably 1.4 to 20, especially 2 to 20) ml/g, and a specific surface area of 0.1 to 500 (preferably 1 to 400) m²/g, and containing, after hydrolysis of the anhydride groups, 0.01 to 14 (preferably 0.02 to 11, especially 0.02 to 10) milliequivalents of acid per gram.
- 20 We also provide a process for the production of a preparation as defined above in which a cross-linked copolymer as defined above is reacted in aqueous solution with a solution of the peptide material to give the desired preparation. Preferably this process
- 25 comprises also the prior step of producing the said cross-linked copolymer by copolymerising, by precipitation or bead polymerisation in a diluent inert to anhydride groups at 20 to 30 200°C in the presence of a free-radical initiator, a mixture comprising:
- A: 0.1 to 50, preferably 2 to 25 per cent of the said at least one anhydride;
- 35 B: 35 to 90, preferably 50 to 85, per cent of the said at least one acrylate; and
- C: 5 to 60, preferably 10 to 50, per cent of the said at least one hydrophilic monomer;
- 40 relative to the total weight of copolymerisable monomers present.
- Finally, we also provide according to this invention a process for carrying out an enzyme-catalysed conversion of a substrate comprising contacting a preparation as defined
- 45 above in which the peptide material is an enzyme with a substrate for the enzyme under conditions where the said conversion occurs.
- In this specification the term "peptide material" means a protein, a protein-containing enzyme or enzyme inhibitor, polypeptide,
- 50 oligopeptide or aminoacid.
- The starting materials required for the production of the preparations of the invention will first be described.
- 55 The copolymers *per se* and a process for their preparation are claimed in Application No. 23601/75, (Serial No. 1413384), divided herefrom.
- 60 Important examples of α,β - monoolefinically unsaturated dicarboxylic acid anhydrides with 4—9 carbon atoms, preferably with 4—5 carbon atoms, which are required to provide residues A are maleic, itaconic and citraconic anhydrides, especially maleic anhydride. Mix-
- tures of these anhydrides can also be used 65 for the copolymerisation.
- The diols and polyols from which the dimethacrylates, polymethacrylates, diacrylates and polyacrylates used to provide residues B in this invention are derived comprise the 70 following categories of compounds:—
- (i) di or polyhydroxy compounds with at least two alcoholic or phenolic, preferably alcoholic, hydroxyl groups;
- (ii) the reaction products of the said di- or 75 polyhydroxy compounds (i) with alkylene oxides having 2 to 8, preferably 2 to 4 carbon atoms, or mixtures of such alkylene oxides, 1 to 10⁴, preferably 1 to 10, alkylene oxide units being added to one 80 mol of the di- or polyhydroxy compound (i). Examples of suitable alkylene oxides are ethylene oxide, propylene oxide, butylene oxide, trimethylene oxide, tetramethylene oxide, bischloromethyl - 85 oxacyclobutane and styrene oxide, ethylene and propylene oxides being preferred;
- (iii) the reaction products of at least one 90 of the alkylene oxides defined in (ii) with a compound having at least two Zerewitinoff-active hydrogen atoms which is not an alcohol or a phenol.
- The di- and polyacrylates and methacrylates of diols and polyols, to be employed 95 according to the invention, can be obtained in accordance with known methods, for example by reaction of the diols and/or polyols with acrylic acid chloride or methacrylic acid chloride in the presence of about equimolar 100 amounts, relative to acid chloride, of tertiary amines such as triethylamine, at temperatures below 20°C, in the presence of benzene (compare German Offenlegungsschrift 1,907,666).
- Possible diols or polyols with at least 2 carbon 105 atoms, preferably 2—12 carbon atoms, are, for example: ethylene glycol, 1,2 - propanediol, 1,3 - propanediol, butanediols, especially 1,4 - butanediol, hexanediols, decanediols, glycerine, trimethylolpropane, pentaerythritol, 110 sorbitol, sucrose and their reaction products with alkylene oxides, as indicated above. Polybis - chloromethyl - oxacyclobutane or polystyrene - oxide are also suitable. Mixtures of diols and polyols can also be employed to 115 produce the acrylates.
- Preferably, diacrylates of diols with 2—4 carbon atoms and/or reaction products of one mol of these diols with 1—10 mols of alkylene oxide with 2—4 carbon atoms or trimethylolpropane trimethacrylate are used. 120
- The dimethacrylates of ethylene glycol, diethylene glycol, triethylene glycol, tetraethylene glycol or those higher polyalkylene glycols that have molecular weights of up to 500, or 125 their mixtures, are particularly advantageous.
- The third group of the copolymerisation components, giving residues C, consists of hydrophilic monomers, by which is meant any

polymerisable, single unsaturated compound which does not fall within the definition of component B, which does not possess any functional groups which are reactive towards anhydride groups, and which forms hydrophilic polymers.

The hydrophilic monomers C preferably possess at least one carboxyl, aminocarbonyl, sulpho or sulphamoyl group, and the amino groups of the aminocarbonyl or sulphamoyl radical can optionally carry as substituents alkyl groups with 1—4 carbon atoms or alkoxymethyl groups with 1—4 carbon atoms in the alkoxy radical.

As examples of these hydrophilic monomers, there may be mentioned acrylic acid, methacrylic acid, maleic acid half-esters with 1—8 carbon atoms in the alcohol radical, N-vinyl lactams such as N-vinylpyrrolidinone, methacrylamide, N-substituted acrylamides and methacrylamides such as N-methyl- and N-methoxymethylacrylamide or methacrylamide and N-acryloyldimethyltaurine.

Depending on the desired hydrophilic character and swellability of the copolymers and on the length of the polyalkylene oxide chain or chains of the polyfunctional acrylic or methacrylamide ester B, the hydrophilic monomers C are added to the monomer mixture in amounts of 5—60% by weight, relative to the total weight of the copolymerisable monomers present.

Apart from affecting the hydrophilic character, the addition of these monomers surprisingly also effects the structure of the polymers, which is of very particular advantage in the production of bead polymers, since here the choice of the diluents for the monomer mixture is greatly restricted by the conditions that they should be insoluble in paraffin hydrocarbons and inert towards anhydride groups.

Because of the range of variation in the composition of the monomer mixture, the hydrophilic character, density of crosslinking, swellability and anhydride group content of the copolymers according to the invention can be adapted to give optimal results in the intended particular end use of the preparation over a very broad range.

If desired it is also possible to add, in addition to the di- and/or polyacrylates methacrylates to be used according to the invention at least one crosslinking agent having at least 2 non-conjugated double bonds (e.g. divinyl adipate, methylene-bis-acrylamide, triacrylformal and triallyl cyanurate) to the monomer mixture in amounts of 0.01—30% by weight, based on the total monomer residues of the copolymer.

The polymerisation can be carried out, for example, in an organic solvent, as a precipitation polymerisation, in which case the polymers start to precipitate shortly after the start of the polymerisation. Inherently, all solvents

which are inert towards anhydride groups are suitable. Particularly advantageous solvents are aliphatic, cycloaliphatic and aromatic hydrocarbons and halogen-substituted hydrocarbons, alkyl-aromatic compounds and carboxylic acid esters.

As examples of suitable organic solvents there may be mentioned: heptane, octane, isooctane, petroleum fractions with boiling points of 60 to 200°C, cyclohexane, benzene, toluene, xylenes, chlorobenzene, dichlorobenzenes, ethyl acetate and butyl acetate.

The solvent used should preferably possess a boiling point of at least 60°C and should be easily removable from the precipitation polymer *in vacuo*. For 1 part of the monomer mixture, about 2—50, preferably 5—20, parts by weight of the solvent are used. The properties of the copolymers, especially the bulk density and the specific surface area, are significantly influenced by the nature and amount of the solvent.

In many cases it is advantageous to use mixtures of the above mentioned solvents or to start the polymerisation in a solvent for the polymer and continuously to add a precipitant for the polymer over the course of the polymerisation. The precipitant can also be added in one or more portions at particular points in time. Furthermore, the monomer mixture can be fed, together with a suitable initiator, as a solution or without solvent, into a previously taken amount of solvent, so that during the polymerisation a uniform low monomer concentration is maintained. Products with swellability, density and specific surface area suited to the particular end use, and with good mechanical stability, having a very wide range of properties, can be produced by using acrylic or methacrylic monomers B of differing hydrophilic character and varying the polymerisation conditions.

The copolymers used according to the invention are preferably produced by suspension polymerisation. The most customary type of bead polymerisation, in which the monomers, optionally with the addition of an organic solvent, are suspended in water, can in this case only be used with little success, since the anhydride hydrolyses very rapidly and the resulting dicarboxylic acid mainly passes into the water phase, only small amounts being incorporated into the polymer. Hence, the suspension polymerisation is preferably carried out in an organic medium. Paraffin hydrocarbons, such as hexane, heptane, octane and higher homologues, cycloaliphatic compounds such as cyclohexane, and paraffin mixtures such as petroleum fractions or paraffin oil, are particularly suitable as the continuous phase. The monomers and the initiator are generally dissolved in a solvent which is immiscible with paraffins and inert towards anhydride groups, such as, for example, acetonitrile, dimethylformamide, dimethylsulph-

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oxide or hexamethylphosphoric acid triamide, and dispersed in the continuous phase, in most cases with addition of at least one dispersing agent. The volume ratio of continuous phase: 5
disperse phase is generally 1:1 to 10:1, preferably 2:1 to 5:1.

In order to stabilise the suspension it is possible to use, for example, glycerine mono- 10
oleate and dioleate, as well as mixtures of these compounds, sorbitan monooleate and trioleate or monostearate and tristearate, poly-
ethylene glycol monoethers with stearyl alco- 15
hol or lauryl alcohol or nonylphenol, poly-ethylene glycol monoesters with oleic acid, stearic acid and other fatty acids with more
than 10 C atoms, and the Na salt of sulpho- 20
succinic acid dioctyl ester. These stabilizing substances are generally employed in amounts of, preferably, 0.1—10% relative to the mono-
mer mixture and are generally dissolved in the hydrocarbon phase. The particle size of 25
the copolymeric product can be reduced either by increasing the speed of stirring or by adding 0.01—2%, relative to monomer, of a
further surface-active substance, for example an alkylsulphonate.

Polymerisation in the process of the inven-
tion is initiated by radical initiators. Suitable 30
initiators are, for example, azo compounds or per-compounds. The most customary azo compound for initiating the polymerisation is azoisobutyronitrile. Possible per-compounds
are mainly diacyl peroxides such as dibenzoyl 35
peroxide or percarbonates such as diisopropyl percarbonate and dicyclohexyl percarbonate, but it is also possible to use dialkyl peroxides, hydroperoxides and redox systems which are
active in organic solvents for the initiation.

The initiators are generally added in 40
amounts of 0.01—10 wt. %, preferably 0.1—3 wt. %, relative to the weight of the mono-
mer mixture.

The polymerisation is generally carried out 45
at temperatures of 20—200°C, preferably 50—100°C, depending on the speed of decomposition of the initiators and, in most
cases, below the boiling point of the solvent, and in the case of bead polymerisations be- 50
low the miscibility temperature of the two phases. Furthermore it is as a rule advantageous
to carry out the polymerisation in an inert atmosphere in the absence of oxygen.

The copolymers obtained by precipitation 55
polymerisation are colourless to pale yellow-coloured powdery substances with bulk
volumes of 1.5—30 ml/g, preferably 2—20 ml/g and specific surface areas of 0.1—500
m²/g, preferably 1—400 m²/g. The content of carboxyl groups determined titrimetrically 60
after hydrolysis of the anhydride groups is about 0.01—14 m-equiv/g, preferably about 0.02—11 m-equiv/g.

The copolymers obtained by suspension 65
polymerisation are white or slightly coloured beads which can in some cases be irregular

in shape and have a diameter of 0.03—3 mm, preferably 0.05—0.5 mm and bulk volumes of 1.4—8 ml/g, preferably 1.4—5 ml/g. Their
carboxyl group content, determined after hydrolysis of the anhydride groups, is 0.01— 70
14 m-equiv/g, preferably 0.02—11 m-equiv/g.

The copolymers used according to the invention contain the copolymerised residues 75
essentially in statistical distribution. Because of their high density of crosslinking, they are insoluble in all solvents. Molecular weights
can therefore not be determined.

The copolymers can swell in water to be- 80
tween 1.1 and 3 times their bulk volume. They are outstandingly suitable for use as carrier resins for fixing substances which can react
with the anhydride groups of the copolymers. They furthermore possess excellent mech- 85
anical stability and hence are not liable to significant losses through abrasion.

The carrier copolymers described above bind all substances which carry a functional 90
group which is capable of reacting with the anhydride group of the polymer. In the case of proteins and peptides these are above all
the terminal amino groups of lysine and the free amino groups of the peptide chain ends.

After its production, the copolymer is com- 95
bined with the peptide material in a second step. In this step, the requisite amount of polymer is introduced into a stirred aqueous
solution of the peptide material at a tempera-
ture of between 0 and 30°C. The weight ratio of peptide materials to carrier resin can be 100
varied within wide limits and be suited to the subsequent end use. Good yields are obtained
for example with a ratio of 1 part by weight of protein to between 4 and 10 parts by 105
weight of polymeric carrier. However, the optimum ratios depend both on the composition and structure of the polymer and on
the nature of the protein.

In the case of numerous enzymes it is also 110
advisable to add stabilisers. As such stabilisers it is possible to use polyethylene gly-
cols or non-ionic wetting agents for reducing denaturation at surfaces, as well as the known
SH-reagents or metal ions in the case of special enzymes. The pH is appropriately kept 115
by means of a pH-stat at the optimum pH-value for the particular bonding. This pH-
value lies in a range of 2 to 9, preferably 5 to 7. When the peptide material is penicillin
acylase it has proved advisable to work at be- 120
tween pH 5.7 and pH 6.8, and with a low salt content, preferably not more than 0.2
mol/litre, in the penicillin acylase solution. In doing so, inorganic bases (for example caustic
alkali solutions) or organic bases (for example tertiary organic amines) must be added in 125
order to keep the pH-value constant. The progress of the reaction is discernible from the
consumption of the amount of base required to keep the pH constant. At room tempera- 130

ture, about 16 hours are required to complete the reaction and at 4°C up to 40 hours are required. Thereafter the copolymer is filtered off and is washed with buffers or salts, in the concentration range of 0.2 to 1 M, in order to dissolve off a small proportion of ionically bound protein.

The bound substance can be determined either by elementary analysis or, in the case of enzymes or inhibitors, by the enzymatic activity or the inhibition of the enzymatic activity. The yields of bound substance depend on the nature of the substance and the composition of the polymer. Thus, for example, aminoacids and low molecular peptides were coupled practically completely; however, even with the proteins the yields of bound enzymatic activity are between about 20 and over 90% of the activity introduced.

According to the state of the art (German Offenlegungsschriften 1,935,711 and 2,008,990, the coupling of proteins is effected in buffer solutions in concentrations of 0.05 M to 0.2 M. If no buffer is used, the proportion of ionically bound protein increases (German Offenlegungsschrift 1,935,711). Surprisingly it was found that with the preparations according to the invention the coupling in a medium which is as free of ions as possible gives maximum yields of enzyme bound by covalent bonds. In this procedure, the pH can be kept constant very accurately at the optimum value by means of a pH-stat.

The use of copolymer-bound enzymes is industrially of particular importance since industrially valuable products can be manufactured with their aid. The prior binding of biological catalysts in accordance with the present process permits them to be isolated completely from the reaction mixture by very simple separation processes and makes it possible to re-use them repeatedly, which is a decisive factor economically. Additionally, in most cases the stability of the sensitive and expensive proteins is decisively improved.

The following gives some examples of the use of the preparations of the invention in carrying out enzyme-catalysed conversions in accordance with the present invention.

The proteases, such as trypsin, chymotrypsin, papain and elastase, can be used, for example, for the production of protein hydrolysis products for microbiological processes. Furthermore, the enzymes can also be used to remove antigenic proteins from pharmaceutically active substances.

Acylases are used industrially for the manufacture of 6 - aminopenicillanic acid from penicillins or for the separation of racemates of acylated aminoacids. Carrier-bound amylase according to the invention can be used for the hydrolytic degradation of starch.

Special hydrolases, such as asparaginase or urease, can be employed therapeutically, in the

copolymer-bound form, in extra-corporal circulation.

These and numerous other possible uses have been described in the literature. See, for example, the summaries in Chem. Eng. News of 15.2.1971, page 86 and Rev. Pure & Appl. Chem. 21, 83 (1971).

A further large field of use of substances fixed to carriers is affinity chromatography. Thus antibodies can be isolated by means of bound antigens or haptens, and conversely antigens can be isolated with fixed antibodies (γ -globulins). Equally, enzymes can be enriched specifically with the aid of bound inhibitors or substrate analogues. For example, it is known to isolate trypsin and chymotrypsin by means of bound vegetable or animal inhibitors. Other examples are the isolation of peptidases on special bound peptides or the isolation of plasmin with lysine bound to a carrier. Summarising works on this field of use have been published by G. Feinstein in Naturwissenschaften 58, 389 (1971) and F. Fried in Chromatographic Reviews 14, 121, (1971).

In order to produce 6 - aminopenicillanic acid (6-APA), penicillins can be split by means of acylases from microorganisms, for example, bacteria, especially *E. coli*, *Erwinia*, *Actinomyces* such as *Streptomyces*, *Micromonospora* and *Nocardia*; fungi such as *Fusarium*; and yeasts.

According to the process of German Patent Specification 1 111 778 for the production of 6-APA, a penicillin G solution is treated with a bacterial sludge which contains the enzyme penicillin acylase (E.C. 3.6.1.11). As a result of the action of the enzyme, the lateral carbonamide grouping of the penicillin is split off without opening the β -lactam ring.

The use of suspensions of micro-organisms has the following disadvantages:

a. The suspension of micro-organisms, in addition to containing the intra-cellular penicillin acylase, contains further proteins and enzymes as well as constituents from the nutrient medium or their transformation products which have been produced during the fermentation. The impurities cannot be completely eluted from the crystalline 6-APA during working up.

b. The suspension of micro-organisms can economically appropriately only be employed once.

c. The suspension of micro-organisms contain impurities and other enzymes which inactivate penicillin and/or 6-APA by opening the β -lactam ring.

d. The suspension only contains small amounts of penicillin acylase. The use of more enzyme material, for example to achieve shorter reaction times and hence better 6-APA yields with a lower content of extraneous products is not possible to practice.

e. The operating yields of 6-APA depend

on the varying formation of penicillin acylase in the individual fermentation batches.

f. The complete removal of suspended micro-organisms requires an additional process step when working up the 6-APA batches and this causes losses in yield. Further purification steps are necessary to remove protein-like impurities which can cause allergic reactions (British Patents 1,169,696); 1,078,847; and 1,114,311).

All disadvantages mentioned are avoided if instead of a suspension of micro-organisms a penicillin acylase is used which is obtained by covalent bonding of the enzyme to a water-insoluble carrier.

Whilst attempts to produce 6-APA by enzymatic splitting of penicillins with carrier-bound penicillin acylase are known (see, on this subject, German Offenlegungsschrift (Published Specification) 1,917,057 and German Offenlegungsschrift 1,907,365) it has not been possible to apply the process described on a technical scale. The reasons for this are firstly the mechanical properties of the carrier material used, which is very vulnerable to abrasion, whilst with moderate process yields only low specific activities of carrier-bound penicillin acylase were achievable.

The insoluble enzyme used in British Patent Specification No. 1,387,460, which was obtained by covalent bonding of the penicillin acylase to a copolymer of acrylamide, N,N'-methylene-bis-acrylamide and maleic anhydride, also has disadvantages for the splitting of penicillin on an industrial scale, since it swells strongly and is mechanically unstable. These disadvantages handicap the repeated reuse of the resin, thus manufactured, on an industrial scale. It has now been found that the disadvantages mentioned are avoided if a preparation according to the invention comprising a penicillin acylase bound to a water-insoluble carrier is used for splitting penicillins.

The splitting of penicillins with carrier-bound penicillin acylase according to the invention can be carried out simply and also on a large industrial scale. In a preferred form of this process, the carrier-bound insoluble enzyme is suspended in a solution containing 75,000—150,000 IU/ml of penicillin [1 mg. of potassium benzylpenicillin G corresponds to 1598 IU (International Units)], for example penicillin G or penicillin V. The enzymatic splitting is carried out at a constant pH-value in the range of 6—9, particularly in the range of the pH optimum of the particular bound penicillin acylase, for example at pH 7.8. To neutralise the acyl radical split off, for example of phenylacetic acid or of phenoxycetic acid, aqueous alkali solutions, for example potassium hydroxide solution or sodium hydroxide solution, or organic amines, preferably triethylamine, are used. The reaction velocity and the completion of the split-

ting can be seen from the consumption of the base. The penicillin acylase catalyses both the splitting of penicillin to give 6-APA and re-synthesis of the penicillins from the splitting products. The equilibrium depends on the pH-value of the medium. At lower pH-value the equilibrium is displaced in favour of the starting product, penicillin. This can be utilised for the trans-acylation of penicillins in the presence of other acyl radicals or for the synthesis of penicillins from 6-APA.

The reaction temperature of the enzymatic splitting is preferably 38°C. At lower temperatures, the activity of the enzyme decreases. If the splitting is carried out, for example, at 25°C, twice as much enzyme as at 38°C has to be employed if the same reaction times are to be achieved.

At a given temperature, the reaction velocity depends on the specific activity and on the amount of the carrier-bound penicillin acylase. Furthermore, the reaction velocity depends on the ratio of the amount of the carrier-bound penicillin acylase to the concentration of the penicillin. A splitting batch with a concentration of 100,000 IU/ml of potassium penicillin G has been completely hydrolysed to 6-APA and phenylacetic acid after 10 hours at pH 7.8 and 38°C if, per unit of penicillin acylase, 3×10^5 units of penicillin G are employed (one enzyme unit (U) is defined as the activity which hydrolyses 1 μ mol of 6-nitro-3-(phenyl-acetyl)-amino-benzoic acid (NIPAB) per minute at 25°C). The proportion of dry enzyme resin is only 0.5—1% of the reaction mixture. If 2 units of penicillin acylase are employed per 10^5 IU of penicillin G, the complete splitting only requires two hours. Even shorter reaction times are also possible when using even more bound penicillin acylase, when using, for example, carrier-bound penicillin acylase prepared from crystallised enzyme.

The carrier-bound penicillin acylase produced according to the invention is preferably in the shape of beads and is distinguished by high mechanical stability and a comparatively high specific gravity. These properties allow it to be employed for prolonged periods of time if it is repeatedly used. These properties furthermore make it possible, in batch processes, to employ intensive stirring and simple separation by centrifuging without loss through mechanical stress, for example through abrasion. Thus, batch processes yield clear filtrates which can, without additional filtration, be processed further in order to isolate the end product, 6-APA. The carrier-bound penicillin acylase produced according to the invention also permits rapid and simple filtration, since, as a result of the mechanical stability, no very fine particles which block the filter surface are produced. The resin offers further advantages in the batch process because of the comparatively high specific

gravity, which causes the resin to settle out rapidly, so that after completion of the process the supernatant solution can easily be siphoned off. This results in a simplification of the conduct of the process, since in the batch process the resin can remain in the reaction vessel and be used directly for the next splitting.

The properties of the polymer furthermore permit the use of the carrier-bound penicillin acylase not only in batch processes but also in continuous processes, for example in reaction columns, where the bead shape permits the requisite high speed of flow through the column.

After separating off the enzyme resin, the 6-APA formed in the enzymatic splitting can be isolated from the reaction solution in accordance with known processes (see, for example, German Patent Specification 1 111 778) and is crystallised at pH 4.3. In the splitting of penicillin, according to the invention, with the carrier-bound penicillin acylase produced according to the invention, substantially higher yields of 6-APA are obtained than when using *E. coli* sludge, but also higher yields than when using the enzyme resin according to British Patent Specification No. 1,387,460. Thus, as is shown in Examples 14 to 17 below, 6-APA has been isolated in a yield of about 90% of theory. The 6-APA thus produced does not contain any proteins as impurities. The 6-APA thus produced also contains practically none of the polymers which can be produced, as by-products in other procedures. Allergic side-effects attributable to such proteins or polymers are impossible with the 6-APA produced according to the invention.

The carrier-bound penicillin acylase produced according to the invention can be used repeatedly over a prolonged period of time. Even thereafter, the enzyme activity is still retained practically completely.

Our pending Application No. 15114/73, (Serial No. 1413381), discloses and claims new preparations generally similar to those of the present invention, but lacking residues C.

The following Examples illustrate the peptide-copolymer preparations according to this invention. Boiling points therein were determined at normal pressure.

Example 1

(a) 70 g of tetraethylene glycol dimethacrylate, 20 g of methacrylic acid, 10 g of maleic anhydride and 1 g of azoisobutyronitrile are dissolved in 1 l of benzene and initially polymerized for 4 hours at 60°C. 1 g of azoisobutyronitrile and 200 ml of petroleum (boiling point 100—140°C) are then added and the polymerisation is carried out for 2 hours at 70°C and 2 hours at 80°C.

The pulverulent polymer is thoroughly

washed with petroleum ether (boiling point 30—50°C) and dried *in vacuo*.

Yield: 96 g

Bulk volume: 8.8 ml/g

Swelling volume in water: 12.4 ml/g

Specific surface area: 8.6 m²/g

Acid content after hydrolysis of the anhydride groups=3.85 m-equiv/g.

(b) 6 g of the carrier copolymer produced according to part (a) above are suspended in a solution of 610 U of penicillin acylase in 150 ml of water. The pH-value is kept at 6.3 by adding 1 N NaOH, using a pH-stat, and the suspension is stirred for 20 hours at 25°C.

The copolymer is then filtered off on a G 3 glass frit and washed with 300 ml of 0.05 M phosphate buffer of pH 7.5, containing 1 M sodium chloride, and with 300 ml of the same buffer without sodium chloride. No further activity can be eluted by further washing. The supernatant liquid and the wash solutions are combined and their enzymatic activity is determined. The enzymatic activity of the moist copolymer is measured in an aliquot amount.

Result:

Enzymatic activities (NIPAB test):

Starting solution 610 U

Supernatant liquid + wash solutions 112 U

Carrier copolymer after the reaction 561 U

(ie 92% of the starting activity)

The enzymatic activity of the penicillin acylase is measured colorimetrically or titrimetrically with 0.002 M of 6 - nitro - 3 - (N - phenylacetyl) - aminobenzoic acid (NIPAB) as the substrate at pH 7.5 and 25°C. The molar extinction co-efficient of the resulting 6 - nitro - 3 - aminobenzoic acid is $E_{405\text{ nm}}=9,090$. 1 unit (U) corresponds to the conversion of 1 μmol of substrate per minute.

(c) 400 mg of the carrier copolymer produced according to part (a) above are suspended in a solution of 40 ml of urease in 32 ml of water. The pH-value is kept constant at pH 6.0 by adding 1 N sodium hydroxide solution, whilst constantly stirring at room temperature. After 16 hours, the reaction is complete and the copolymer is filtered off and washed with buffer as in part (b).

Result:

Enzymatic activity:

Starting solution 168 U

Supernatant liquid + wash solutions 64.5 U

Carrier copolymer after the reaction 87 U

(ie 52% of the starting activity)

The enzymatic activity of the urease is determined titrimetrically with 0.17 M urea as the substrate, at 25°C and pH 6.1. 1 unit(U) corresponds to the amount of enzyme which consumes 1 μ mol of urea, that is to say 2 μ mol of hydrochloric acid, per minute.

- 5 (d) 500 mg of the carrier copolymer produced according to Example 1 a) are suspended in a solution of 100 mg of trypsin in 32 ml of 0.02 M calcium chloride. The pH-value is kept constant at pH 6.3 by addition of 1 N sodium hydroxide solution whilst constantly stirring at 4°C. After 16 hours the copolymer is filtered off and washed with
10 buffer as in part (b).
15

Result:

Enzymatic activity:

- | | |
|--------------------------------------|--------|
| Starting solution | 110 U |
| Supernatant liquid + wash solutions | 15.6 U |
| Carrier copolymer after the reaction | 64 U |

(ie 58% of the starting activity)

- 25 The enzymatic activity was determined colorimetrically according to Tuppy. Z. Physiol. Chem. 329 (1962) 278, with benzoyl - arginine - p - nitroanilide (BAPNA) as the substrate. 1 unit (U) corresponds to the splitting of 1 μ mol of substrate per minute
30 at 25°C and pH 7.8.

Example 2

- (a) A solution of 80 g of tetraethylene glycol dimethacrylate 10 g of methacrylic acid, 10 g of maleic anhydride and 1 g of azoisobutyronitrile in 1 l of benzene is polymerised for 4 hours at 60°C, 2 hours at 70°C and 1 hour at 80°C, whilst stirring slowly. The polymer is filtered off, stirred three times in benzene, washed with petroleum ether and
40 dried *in vacuo*.

Yield: 70 g

Bulk volume: 7.0 ml/g

Swelling volume in water: 8.2 ml/g

Specific surface area: 5.3 m²/g

- 45 Acid content after hydrolysis of the anhydride groups=2.55 m-equiv/g.

- (b) 1 g of the polymer produced according to part (a) of this Example is reacted, analogously to Example 1 b), with penicillin acylase in 33 ml of water at pH 6.3.
50

Result:

Enzymatic activities (NIPAB test):

- | | |
|--------------------------------------|-------|
| Starting solution | 118 U |
| Supernatant liquid + wash solution | 21 U |
| Carrier copolymer after the reaction | 82 U |

(ie 69% of the starting activity)

Example 3

- (a) 60 g of tetraethylene glycol dimethacrylate, 30 g of methacrylic acid, 10 g of maleic anhydride and 1 g of azoisobutyronitrile are dissolved in 300 ml of acetonitrile. This solution is suspended in 1 l of petroleum (boiling point 100—140°C) which contains 5 g of a mixture of glycerine monooleate and glycerine dioleate and is polymerised for 22 hours at 60°C.
60
65

The polymer beads are filtered off, suspended three times in benzene and subsequently twice in petroleum ether (boiling point 30—50°C) and dried *in vacuo*.
70

Yield: 94 g of white spheres

Bulk volume: 4.4 ml/g

Swelling volume in water: 5.5 ml/g

Specific surface area: 6.6 m²/g

Mean particle diameter: ~200 μ

Acid content after hydrolysis of the anhydride groups=4.3 m-equiv/g.
75

- (b) 1 g of the polymer produced according to Example 3a) is reacted analogously to Example 1 b) with penicillin acylase in 33 ml of water at pH 6.3.
80

Result:

Enzymatic activity (NIPAB test):

- | | |
|--------------------------------------|-------|
| Starting solution | 118 U |
| Supernatant liquid + wash solutions | 43 U |
| Carrier copolymer after the reaction | 96 U |

(ie 81% of the starting activity)
90

Example 4

- (a) A solution of 2,100 g of tetraethylene glycol dimethacrylate, 600 g of methacrylic acid, 300 g of maleic anhydride and 30 g of azoisobutyronitrile in 7.5 l of acetonitrile is suspended in 21 l of petroleum (boiling point 100—140°C) in which 150 g of a mixture of glycerine monooleate and glycerine dioleate have been dissolved, and is polymerised for 1 hour at 50°C and 20 hours at 60°C.
95
100

The bead polymer is filtered off, twice stirred with toluene and once with petroleum ether (boiling point 30—50°C) and dried at 60°C.
105

Yield: 2.95 kg

Bulk volume: 4.7 ml/g

Swelling volume in water: 5.4 ml/g

Mean particle diameter: approx. 0.3 mm

Acid content after hydrolysis of the anhydride groups=4.0 m-equiv/g.
110

- (b) 1 g of the polymer produced according to part (a) of this Example is reacted as described in Example 1 b) with penicillin acylase in 33 ml of water at pH 6.3.
115

Result:		Example 6	
Enzymatic activity (NIPAB test):		(a) A solution of 50 g of ethylene glycol dimethacrylate, 40 g of methacrylic acid, 10 g of maleic anhydride and 1 g of azoisobutyronitrile in 300 ml of acetonitrile is suspended in 1 l of petroleum (boiling point 100—140°C) in which 5 g of a mixture of glycerine monooleate and glycerine dioleate are dissolved, and is polymerised for 20 hours at 60°C. The bead polymer is filtered off, suspended three times in benzene and twice in petroleum ether (boiling point 30—50°C) and dried <i>in vacuo</i> at 50°C.	60
5	In the starting solution	107 U	
	Supernatant liquid and wash solutions	27 U	
	Carrier copolymer after the reaction	67 U	
	(ie 63% of the starting activity)		65
10	(c) 20 g of the polymer produced according to part (a) of this Example are introduced into 600 ml of a solution of 1.0 g of non-specific elastase from pig pancreas at room temperature, whilst stirring. At the same time the pH is kept constant at 5.8. After a reaction time of 16 hours, the copolymer is filtered off and worked-up in the manner described in Example 1 b).		
15	Result:		70
	Enzymatic activity (casein test):		
20	Starting solution	2,180 U	
	Supernatant liquid + wash solutions	993 U	
	Carrier copolymer after the reaction	1,225 U	
25	(ie 56% of the starting activity)		75
	The enzymatic activity of the non-specific elastase is determined titrimetrically with casein as the substrate (concentration 11.9 mg/ml) at pH 8.0 at 25°C. 1 unit (U) corresponds to a consumption of 1 μ mol of potassium hydroxide solution per minute.		
30	Example 5		
	A solution of 80 g of tetraethylene glycol dimethacrylate, 10 g of methacrylic acid, 10 g of maleic anhydride and 1 g of azoisobutyronitrile in 300 ml of acetonitrile is polymerised as described in Example 3 a).		
40	Yield: 92 g of white, egg-shaped particles		
	Bulk volume: 2.5 ml/g		
	Swelling volume in water: 3.2 ml/g		
	Specific surface area: 1.7 m ² /g		
	Mean particle diameter: 125 μ		
	Acid content after hydrolysis of the anhydride groups=3.5 m-equiv/g.		
45	(b) 1 g of the polymer produced according to part (a) of this Example is reacted as described in Example 1b) with penicillin acylase in 33 ml of water at pH 6.3.		
50	Result:		80
	Enzymatic activity (NIPAB test):		
	Starting solution	118 U	
	Supernatant liquid and wash solutions	29 U	
	Carrier copolymer after the reaction	55 U	
	(ie 47% of the starting activity)		85
	Example 7		
	(a) A solution of 50 g of trimethylolpropane trimethacrylate, 30 g of methacrylic acid, 20 g of maleic anhydride and 1 g of azoisobutyronitrile in 300 ml of acetonitrile is polymerised as described in Example 6(a).		90
	Yield: 86 g		
	Bulk volume: 2.0 ml/g		95
	Swelling volume in water: 2.2 ml/g		
	Mean particle diameter: ~30 μ		
	Acid content after hydrolysis of the anhydride groups=4.1 m-equiv/g.		
	(b) 1 g of the copolymer produced according to Example 7 a) is added to a solution of 100 mg of lysine in 32 ml of water at room temperature, with constant stirring. The pH-value is kept constant at 6.3. After 16 hours, the copolymer is filtered off, suspended in 50 ml of 1 M sodium chloride solution, filtered off and rinsed with 100 ml of water. The residue is dried <i>in vacuo</i> at 100°C and the nitrogen content is determined by Kjeldhal's method.		100
55	Result:		105
	Dry weight: 860 mg		
	Nitrogen content: 1.5%, corresponding to a content of 65 mg of the lysine in the copolymer. This represents 65% of the amount of lysine employed.		110
	Result:		115
	Enzymatic activity (NIPAB test):		
	Starting solution	118 U	
	Supernatant liquid + wash solutions	34 U	
	Carrier copolymer after the reaction	61 U	
	(ie 52% of the starting activity)		

Example 8

The following were polymerised as described in Example 6(a) (variation of the amount of the maleic anhydride):

Experiment No.	a	b	c	d	e	f	g
5	TGDM(g)	75	70	65	60	55	45
	MAA (g)	20	20	20	20	20	20
	MA (g)	5	10	15	20	25	35
	AIBN (g)	1	1	1	1	1	1
	Acetonitrile (ml)	250	250	250	250	250	250
10	Petroleum (ml)	1,000	1,000	1,000	1,000	1,000	1,000
	Emulsifier (g)	5	2.5	5	5	5	5
	Yield (g)	95	93	86	88	80	70
	V _s (ml/g)	3.4	3.4	3.6	2.6	3.0	2.5
	V _o (ml/g)	4.4	6.0	4.5	3.5	4.0	3.4
15	Acid (meq./g)	3.1	4.2	3.6	3.8	3.9	4.3

Notes:

TGDM=Tetraethylene glycol dimethacrylate

MAA=Methacrylic acid

MA=Maleic anhydride

20 AIBN=Azoisobutyronitrile

Emulsifier=Mixture of glycerine monooleate and glycerine dioleate

V_s=Bulk volume

V_o=Swelling volume in water

Acid=Acid content after hydrolysis of the anhydride groups.

25 The resulting copolymers can all have protein material bound thereto to form preparations according to this invention.

Example 9

30 (a) A solution of 70 g of tetraethylene glycol dimethacrylate 20 g of acrylic acid, 10 g of maleic anhydride and 1 g of azoisobutyronitrile in 1 l of benzene is warmed for 4 hours at 60°C, 2 hours at 70°C and 2 hours at 80°C, with exclusion of air and whilst stirring slowly. The finely divided precipitation polymer is suspended three times in benzene and twice in acetonitrile, filtered off and dried *in vacuo*.

Yield: 94 g

40 Bulk volume: 6.8 ml/g

Swelling volume in water: 7.6 ml/g

Acid content after hydrolysis of the anhydride groups=1.64 m-equiv/g

45 (b) 1 g of the polymer produced according to part (a) of this Example is reacted as described in Example 1(b), with penicillin acylase in 32 ml of water at pH 6.3.

Result:

Enzymatic activity (NIPAB test):

50 Starting solution 107 U

Supernatant liquid + wash solution 26 U

Carrier copolymer after the reaction 38 U

55 (ie 36% of the starting activity)

Example 10

(a) A solution of 50 g of tetraethylene

glycol dimethacrylate, 40 g of N - vinylpyrrolidone, 10 g of maleic anhydride and 1 g of dicyclohexyl percarbonate in 200 ml of acetonitrile is suspended in 1 l of petroleum (boiling point 100—140°C) which contains 5 g of a mixture of glycerine monooleate and glycerine dioleate and is initially warmed to 50°C for 18 hours, whilst stirring. A further 1 g of the initiator is then added and the mixture is stirred for a further 8 hours at 60°C. The polymer spheres are thoroughly washed with benzene and petroleum ether and dried *in vacuo*.

Yield: 54 g of clear beads

Bulk Volume: 1.6 ml/g

Swelling volume in water: 2.4 ml/g

Mean particle diameter: ~0.3 mm

Acid content after hydrolysis of the anhydride groups= 3.3 m-equiv/g

Nitrogen content: 3.4% = 27% of N - vinylpyrrolidone

(b) 1 g of the polymer produced according to part (a) of this Example is reacted as described in Example 1 b) with penicillin acylase in 33 ml of water at pH 6.3.

Result:

Enzymatic activity (NIPAB test):

Starting solution 107 U 85

Supernatant liquid + wash solutions 26 U

Carrier copolymer after the reaction 82 U

(ie 77% of the starting activity)

90

Example 11

A solution of 70 g of tetraethylene glycol dimethacrylate, 20 g of methacrylamide, 10 g of maleic anhydride, 1 g of azoisobutyronitrile and 200 ml of acetonitrile is suspended in petroleum and polymerised as described in Example 5 a).

Yield: 93 g

Bulk volume: 2.0 ml/g

Swelling volume in water: 3.3 ml/g

Acid content after hydrolysis of the anhydride groups: 1.7 m-equiv/g

(b) 1 g of the polymer produced as described in part (a) of this Example is reacted as described in Example 1 b) with penicillin acylase in 32 ml of water at pH 6.3.

Result:

Enzymatic activity (NIPAB test):

Starting solution 107 U

Supernatant liquid and wash solutions 42 U

Carrier copolymer after the reaction 33 U

(ie 31% of the starting activity)

Example 12

(a) A solution of 60 g of tetraethylene glycol dimethacrylate, 30 g of N - methoxymethyl - acrylamide, 10 g of maleic anhydride and 1 g of azobisbutyronitrile in 200 ml of acetonitrile is suspended in 1 l of petroleum in which 5 g of a mixture of glycerine monooleate and glycerine dioleate are dissolved, and is polymerised for 20 hours at 60°C.

The bead polymer is filtered off, suspended three times in ethyl acetate and dried *in vacuo* at 50°C.

Yield: 97 g

Bulk volume: 1.6 ml/g

Swelling volume in water: 3.1 ml/g

Mean particle diameter: ~0.3 mm

(b) 1 g of the polymer produced according to part (a) of this Example is reacted as described in Example 1 b) with penicillin acylase in 32 ml of water at pH 6.3.

Result:

Enzymatic activity (NIPAB test):

Starting solution 107 U

Supernatant liquid + wash solutions 49 U

Carrier copolymer after the reaction 25 U

(ie 23% of the starting activity)

Example 13

(a) If, in the procedure of Example 12(a), 30 g of N - methoxymethylmethacrylamide are employed instead of N - methoxymethyl - acrylamide, the following result is obtained:

Yield: 94 g

Bulk volume: 2.4 ml/g

Swelling volume in water: 3.9 ml/g

Mean particle diameter: ~0.6 mm

(b) 1 g of the polymer produced according to part (a) of this Example is reacted as described in Example 1 b) with penicillin acylase in 32 ml of water at pH 6.3.

Result:

Enzymatic activity (NIPAB test):

Starting solution 107 U

Supernatant liquid and wash solutions 60 U

Carrier copolymer after the reaction 24 U

(ie 22% of the starting activity)

Example 14

76.1 kg (moist weight) of carrier-bound penicillin acylase produced according to Example 4(b) having an activity of 737,000 U(NIPAB test) and 125 kg of potassium penicillin G (purity 98%) are successively added to 2,000 l of water and the mixture is stirred at 38°C. The pH value of the reaction batch is kept constant at 7.8 by continuous addition of triethylamine. After 8 hours, no further triethylamine is taken up. The carrier-bound penicillin acylase is centrifuged off, rinsed with 60 l of water and 80 l of 0.2 M phosphate buffer of pH 6.5, and re-employed for further splitting batches. The filtrate, including the wash water, is concentrated to 300 l *in vacuo*. The 6-APA is precipitated by addition of half-concentrated hydrochloric acid at the isoelectric point at pH 4.3 in the presence of 200 l of methyl isobutyl ketone. After one hour the product is filtered off and rinsed with 200 l of water and then with 200 l of acetone. It is dried *in vacuo* at 40°C; melting point 208°C; yield 66.7 kg, representing 91.9% of theory; purity 98%.

The carrier-bound penicillin acylase was successively employed for a total of 30 batches. Even after 30 splitting reactions, the carrier-bound penicillin acylase has not been consumed. The reaction time does not change. Working up took place as described above. The yields of 6-APA achieved are listed below:

	1st splitting reaction	91.9% of theory	16th	"	"	90.2%	"	"	
	2nd	91.4%	"	"	"	90.3%	"	"	
	3rd	91.6%	"	"	"	89.9%	"	"	
	4th	91.0%	"	"	"	89.8%	"	"	
5	5th	91.2%	"	"	"	89.1%	"	"	20
	6th	90.3%	"	"	"	91.7%	"	"	
	7th	91.5%	"	"	"	91.9%	"	"	
	8th	90.9%	"	"	"	91.6%	"	"	
	9th	91.9%	"	"	"	91.1%	"	"	
10	10th	90.7%	"	"	"	91.8%	"	"	25
	11th	91.2%	"	"	"	90.7%	"	"	
	12th	90.1%	"	"	"	91.7%	"	"	
	13th	90.9%	"	"	"	91.4%	"	"	
	14th	90.7%	"	"	"	90.8%	"	"	
15	15th	91.0%	"	"	"	90.3%	"	"	30
Average=90.95% of theory									

Example 15

- 330 g of carrier-bound penicillin acylase produced according to Example 1, having an enzymatic activity of 3,410 U (NIPAB test) and 129 g of potassium penicillin G are successively added to 2,000 ml of water and stirred at 38°C and pH 7.8 as described in Example 14. The penicillin G is completely split to 6-APA and phenylacetic acid over the course of 2 hours. The 6-APA is isolated as described in Example 14. The carrier-bound penicillin acylase is employed twenty times in succession. Even after the twentieth splitting, the reaction time does not have to be extended to achieve complete splitting.

Yields of 6-APA:

	1st splitting reaction	67.5 g (90.1% of theory)
	2nd	68.4 g (91.1% " ")
50	3rd	68.7 g (91.9% " ")
	4th	68.6 g (91.5% " ")
	5th	68.4 g (91.0% " ")
	6th	68.1 g (90.8% " ")
	7th	68.7 g (91.6% " ")
55	8th	68.4 g (91.2% " ")
	9th	68.5 g (91.5% " ")
	10th	68.1 g (90.9% " ")
	11th	68.5 g (91.3% " ")
	12th	68.6 g (91.7% " ")
60	13th	68.5 g (91.3% " ")
	14th	68.1 g (90.8% " ")
	15th	68.3 g (91.1% " ")
	16th	67.8 g (90.5% " ")
	17th	68.0 g (90.6% " ")
65	18th	67.4 g (89.9% " ")
	19th	67.5 g (90.0% " ")
	20th	66.8 g (89.1% " ")

Average=90.89% of theory

Example 16

- 120 g of carrier-bound penicillin acylase (moist weight) according to Example 4, with an activity of 1,160 U (NIPAB test) and 120 g of potassium penicillin G, are introduced into 1,300 ml of water and stirred for 9 hours at 38°C and pH 7.8, as described in Example 14. The mixture is worked up as described in Example 14. The carrier-bound penicillin acylase is employed five times in succession for the enzymatic splitting reaction. The yields of 6-APA achieved are listed below:

	1st splitting reaction	61.2 g (87.6% of theory)
	2nd	62.0 g (88.9% " ")
	3rd	63.3 g (90.6% " ")
85	4th	62.7 g (89.9% " ")
	5th	63.1 g (90.4% " ")

Example 17

290 g of carrier-bound penicillin acylase according to Example 4, having an enzymatic activity of 2,803 U (NIPAB test) and 138 g of potassium penicillin V are added to 2,000 ml of water and stirred for 9 hours at 38°C. The pH-value is kept constant at 7.8 by continuous addition of triethylamine. The mixture is worked up as described in Example 1. Yields of 6-APA 64.3 g (86.1% of theory); purity 97.4%.

WHAT WE CLAIM IS:—

1. A water-insoluble preparation comprising a peptide material (as hereinbefore defined) bound to a cross-linked copolymer comprising the following copolymerised residues (percentages being by weight of the cross-linked copolymer):
 - A: 0.1 to 30 per cent of at least one α,β -monoolefinically unsaturated dicarboxylic acid anhydride having 4 to 9 carbon atoms.
 - B: 35 to 90 per cent of at least one di- and/or polyacrylate or methacrylate of a diol and/or a polyol (as hereinbefore defined); and
 - C: 5 to 60 per cent of at least one hydrophilic monomer (as hereinbefore defined);
- the copolymer having a bulk volume of 1.4 to 30 ml/g and a specific surface area of 0.1 to 500 m²/g, and containing, after hydrolysis of the anhydride groups, 0.01 to 14 milliequivalents of acid per gram.
2. A preparation according to claim 1 in which the copolymer comprises also copolymerised residues derived from 0.01 to 30 per cent by weight of the cross-linked copolymer, of a crosslinking agent having at least 2 non-conjugated double bonds.
3. A preparation according to claim 1 or 2 comprising 2 to 20 per cent by weight of residues A.
4. A preparation according to claim 1, 2 or 3 in which the copolymer contains 50 to 80 per cent by weight of residues B.
5. A preparation according to any preceding claim in which the copolymer contains 10 to 50 per cent by weight of residues C.
6. A process according to any preceding claim in which the copolymer has a bulk volume of 1.4 to 20 ml/g.
7. A preparation according to any preceding claim in which the copolymer has a specific surface area of 1 to 400 m²/g.
8. A preparation according to any preceding claim in which the copolymer contains, after hydrolysis of the anhydride groups, 0.02 to 10 m-equiv of acid per gram.
9. A preparation according to any preceding claim in which the copolymerised residues A of the copolymer are derived from at least one anhydride selected from maleic, itaconic and citraconic anhydrides, and the copolymer-

ised residues B of the copolymer are residues of at least one acrylate selected from diacrylates and dimethacrylates of diols having 2 to 4 carbon atoms, dimethacrylates of reaction products of 1 mol of diols having 2 to 4 carbon atoms with 1 to 10 mols of at least one alkylene oxide with 2 to 4 carbon atoms, and trimethylolpropane trimethacrylate, and the copolymerised units of component C of the copolymer are derived from at least one hydrophilic monomer containing a carboxyl, aminocarbonyl, sulpho or sulphamoyl group.

10. A preparation according to claim 9 in which the copolymerised residues A of the copolymer are of maleic anhydride, the copolymerised residues B of the copolymer are selected from residues of ethylene glycol, diethylene glycol, triethylene glycol, and tetraethylene glycol dimethacrylates, trimethylolpropane trimethacrylates and mixtures thereof, and the copolymerised residues C are selected from residues of methacrylic acid, acrylic acid, methacrylamide, N - methoxymethylacrylamide, N - methoxymethylmethacrylamide, N - vinylpyrrolidone, and mixtures thereof.

11. A preparation according to any preceding claim in which the peptide material is an enzyme.

12. A preparation according to claim 11 in which the enzyme is penicillin acylase.

13. A preparation according to any of claims 1 to 10 in which the peptide material is an enzyme inhibitor.

14. Water-insoluble preparations comprising a peptide material bound to a cross-linked copolymer, substantially as hereinbefore described in any of the Examples.

15. A process for the production of a preparation according to any of claims 1 to 10 in which a cross-linked copolymer as defined in any of claims 1 to 10 is reacted in aqueous solution with a solution of the peptide material to give the desired preparation.

16. A process according to claim 15 comprising also the step of producing the said cross-linked copolymer by copolymerising, by precipitation or bead copolymerisation in a diluent inert to anhydride groups at 20 to 200°C in the presence of a free-radical initiator a mixture comprising:

- A: 0.1 to 50 per cent of at least one α,β -monoolefinically unsaturated dicarboxylic acid anhydride having 4 to 9 carbon atoms; and
- B: 35 to 90 per cent of at least one di- and/or polyacrylate or methacrylate of a diol and/or a polyol (as hereinbefore defined); and
- C: 5 to 60 per cent of at least one hydrophilic monomer (as hereinbefore defined);

relative to the total weight of copolymerisable monomers present.

17. A process according to claim 16 in



Example 17

290 g of carrier-bound penicillin acylase according to Example 4, having an enzymatic activity of 2,803 U (NIPAB test) and 138 g of potassium penicillin V are added to 2,000 ml of water and stirred for 9 hours at 38°C. The pH-value is kept constant at 7.8 by continuous addition of triethylamine. The mixture is worked up as described in Example 1. Yields of 6-APA 64.3 g (86.1% of theory); purity 97.4%.

WHAT WE CLAIM IS:—

1. A water-insoluble preparation comprising a peptide material (as hereinbefore defined) bound to a cross-linked copolymer comprising the following copolymerised residues (percentages being by weight of the cross-linked copolymer):
 - A: 0.1 to 30 per cent of at least one α,β -monoolefinically unsaturated dicarboxylic acid anhydride having 4 to 9 carbon atoms.
 - B: 35 to 90 per cent of at least one di- and/or polyacrylate or methacrylate of a diol and/or a polyol (as hereinbefore defined); and
 - C: 5 to 60 per cent of at least one hydrophilic monomer (as hereinbefore defined);
- the copolymer having a bulk volume of 1.4 to 30 ml/g and a specific surface area of 0.1 to 500 m²/g, and containing, after hydrolysis of the anhydride groups, 0.01 to 14 milliequivalents of acid per gram.
2. A preparation according to claim 1 in which the copolymer comprises also copolymerised residues derived from 0.01 to 30 per cent by weight of the cross-linked copolymer, of a crosslinking agent having at least 2 non-conjugated double bonds.
3. A preparation according to claim 1 or 2 comprising 2 to 20 per cent by weight of residues A.
4. A preparation according to claim 1, 2 or 3 in which the copolymer contains 50 to 80 per cent by weight of residues B.
5. A preparation according to any preceding claim in which the copolymer contains 10 to 50 per cent by weight of residues C.
6. A process according to any preceding claim in which the copolymer has a bulk volume of 1.4 to 20 ml/g.
7. A preparation according to any preceding claim in which the copolymer has a specific surface area of 1 to 400 m²/g.
8. A preparation according to any preceding claim in which the copolymer contains, after hydrolysis of the anhydride groups, 0.02 to 10 m-equiv of acid per gram.
9. A preparation according to any preceding claim in which the copolymerised residues A of the copolymer are derived from at least one anhydride selected from maleic, itaconic and citraconic anhydrides, and the copolymer-

ised residues B of the copolymer are residues of at least one acrylate selected from diacrylates and dimethacrylates of diols having 2 to 4 carbon atoms, dimethacrylates of reaction products of 1 mol of diols having 2 to 4 carbon atoms with 1 to 10 mols of at least one alkylene oxide with 2 to 4 carbon atoms, and trimethylolpropane trimethacrylate, and the copolymerised units of component C of the copolymer are derived from at least one hydrophilic monomer containing a carboxyl, aminocarbonyl, sulpho or sulphamoyl group.

10. A preparation according to claim 9 in which the copolymerised residues A of the copolymer are of maleic anhydride, the copolymerised residues B of the copolymer are selected from residues of ethylene glycol, diethylene glycol, triethylene glycol, and tetraethylene glycol dimethacrylates, trimethylolpropane trimethacrylates and mixtures thereof, and the copolymerised residues C are selected from residues of methacrylic acid, acrylic acid, methacrylamide, N-methoxymethylacrylamide, N-methoxymethylmethacrylamide, N-vinylpyrrolidone, and mixtures thereof.

11. A preparation according to any preceding claim in which the peptide material is an enzyme.

12. A preparation according to claim 11 in which the enzyme is penicillin acylase.

13. A preparation according to any of claims 1 to 10 in which the peptide material is an enzyme inhibitor.

14. Water-insoluble preparations comprising a peptide material bound to a cross-linked copolymer, substantially as hereinbefore described in any of the Examples.

15. A process for the production of a preparation according to any of claims 1 to 10 in which a cross-linked copolymer as defined in any of claims 1 to 10 is reacted in aqueous solution with a solution of the peptide material to give the desired preparation.

16. A process according to claim 15 comprising also the step of producing the said cross-linked copolymer by copolymerising, by precipitation or bead copolymerisation in a diluent inert to anhydride groups at 20 to 200°C in the presence of a free-radical initiator a mixture comprising:

A: 0.1 to 50 per cent of at least one α,β -monoolefinically unsaturated dicarboxylic acid anhydride having 4 to 9 carbon atoms; and

B: 35 to 90 per cent of at least one di- and/or polyacrylate or methacrylate of a diol and/or a polyol (as hereinbefore defined); and

C: 5 to 60 per cent of at least one hydrophilic monomer (as hereinbefore defined); relative to the total weight of copolymerisable monomers present.

17. A process according to claim 16 in

- which the copolymerisation is carried out by precipitation polymerisation in an organic solvent, the organic solvent being present in a proportion of 5 to 20 parts by weight of solvent per part of monomer by weight.
18. A process according to claim 16 in which the copolymerisation is carried out by suspension polymerisation in an organic medium, the monomers and initiator being dissolved in an organic solvent which is immiscible with paraffins and inert towards anhydride groups, and dispersed in a continuous phase comprising a paraffinic hydrocarbon, the volume ratio of continuous phase to disperse phase between 2:1 and 5:1.
19. A process according to any of claims 15 to 18 for the production of a preparation according to claim 12, in which the copolymer is reacted with an aqueous penicillin acylase solution at pH 5.7 to 6.8.
20. A process according to claim 19 in which the salt content of the penicillin acylase solution is not more than 0.2 mol/litre.
21. A process for the production of a preparation according to any of claims 1 to 10, substantially as hereinbefore described in any of the Examples.
22. A water-insoluble preparation comprising a peptide material bound to a cross-linked copolymer, whenever produced by the process of any of claims 15 to 21.
23. A process for carrying out an enzyme-catalysed conversion of a substrate comprising contacting a preparation according to claim 11 with a substrate for the enzyme under conditions where the said conversion occurs.
24. A process according to claim 23 in which the said conversion is a hydrolytic splitting.
25. A process according to claim 24 in which the substrate is a penicillin, the enzyme is penicillin acylase, and the conversion is a hydrolytic splitting of the penicillin to give 6 - amino penicillanic acid.
26. A process according to claim 25 in which the penicillin is in the form of an aqueous solution containing 75,000 to 15,000 IU/ml of penicillin and having a pH of about 7.8.
27. A process for carrying out an enzyme-catalysed conversion of a substrate, substantially as hereinbefore described in the Examples.
28. The product of a process according to any of claims 23 to 27.

For the Applicant,
CARPMAELS & RANSFORD,
Chartered Patent Agents,
43 Bloomsbury Square,
London, WC1A 2RA

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