

The invention concerns a process for the determination of a specifically bindable protein substance according to the immunoassay principle, whereby one of the partners of the substance pair specifically bind-
5 able with one another is present bound to a solid phase, as well as a carrier material suitable therefor.

For the determination of a specifically bindable substance, one frequently uses processes according to the immunoassay principle. One of the partners of a
10 substance pair specifically bindable with one another is thereby reacted with the receptor specific for it which is labelled in per se known manner. The conjugate of these two substances can then be reacted with a receptor which is specific for the conjugate or for
15 one of the two parts of the conjugate. There are many variations for these immunological processes. It is thereby advantageous when one of the receptors is present bound to a solid phase. This makes easier the separation of reaction components present bound and
20 non-bound. For the determination of the specifically bindable substance, there is then measured the amount of labelled reaction partner bound to the solid phase or of labelled reaction partner present in the solution and related in per se known manner to the amount of
25 reaction component to be determined.

As solid phases, in the case of the immunological processes, there are usually used synthetic material test tubes or microtitre plates on the inner surface of which is fixed the reaction partner or spheroids on
30 the outer surfaces of which is fixed the reaction partner. These synthetic material test tubes, microtitre plates or spheroids usually consist of relatively inert synthetic material so that the binding of the reaction partner gives rise to difficulties. Further-
35 more, the binding of the specific reaction partner to

the surface in question must take place in such a manner that it does not lose the ability of specific binding to the substance specifically bindable with it. For this reason, the binding of the reactive partner to the solid phase mostly takes place adsorptively. Therefore, it has already been suggested to bring about the fixing of the reaction partner to the solid phase via a coupling agent which brings about the binding. Care must thereby also again be taken that the binding of the reaction partner to the binding agent does not destroy the specifically reacting region of the molecule or that the reaction partner is so bound that its reactive place is facing away from the solid phase towards the binding component. Furthermore, in DE-OS 25 33 701, it is suggested, in order to achieve a better binding, to cross-link the individual immunologically effective proteins and then to absorb on polystyrene spheroids. As further possibility, it is stated in this literature reference simultaneously to cross-link an inert protein with the protein with immunological properties so that a cross-linked product results of inert and active protein which is then again adsorbed on polystyrene spheroids. However, depending upon chosen reaction conditions, this type of cross-linking leads to differing, non-reproducible cross-linkages with variable proportions of non-cross-linked protein, as well as of protein which has become insoluble. Furthermore, due to the differing degree of cross-linking, products result with differing binding properties. A similar process is described in EP-A1 122 209 and also displays the same disadvantages. Thus, all these known processes do not satisfy and still do not lead to an optimal adhesion of the specifically bindable substance and are of little suitability for the reproducible preparation of coated solid phases.

Therefore, it was the object of the invention to provide a process which reproducibly improves the adhesion of the specifically bindable substance to the solid phase and makes available a carrier material
5 suitable therefor. Since, in the case of many immunological processes, one works with the addition of detergents in order to avoid turbidities, it was also the object of the invention so to improve the adhesion that, even in the presence of detergents, the bound,
10 specifically bindable substance is not dissolved off.

This object is achieved by a process for the preparation of a specifically bindable protein substance bound to an insoluble carrier material, especially for use in a heterogeneous analysis process, according to
15 the immunoassay principle, which is characterised in that one couples a soluble protein with a molecular weight above about 500,000, which is more hydrophobic than the specifically bindable substance, to the specifically bindable substance and then adsorbs the
20 conjugate of reaction partner and protein on a hydrophobic solid phase.

The specifically bindable substance fixed in this way to a solid phase displays an improved adhesion. The binding is also stable with regard to detergents.
25 In the case of the production of calibration curves which are necessary for the evaluation in the case of many immunological processes, the solid phase-bound, specifically bindable substance according to the invention gives steeper calibration curves, which
30 results in an increase of the exactitude.

As further advantage of the process according to the invention, it is possible to control the bound amount more exactly. Since the adhesion is significantly better than in the case of the previously known
35 processes, the amount of specific protein which must be used is also smaller.

For the choice of soluble proteins suitable according to the invention, there must be determined the molecular weight, as well as the hydrophobicity, in comparison with the corresponding value for the specifically bindable substance. The molecular weight is determined by methods known to the expert.

A comparison of the hydrophobicity between soluble protein and specifically bindable substance can also take place by methods known to the expert. Suitable methods are, for example, the comparison

- of the fluorescent extinction after binding to coloured materials (Biochem. Biophys. Acta, 624 (1980) 13-20)
- the elution behaviour in the case of hydrophobic chromatography (Biochem. Biophys. Acta, 576, (1979), 269-279)
- the surface tension (Biochem. Biophys. Acta, 670 (1981), 64-73)
- the retention times in the case of hydrophobic interaction chromatography (HIC) (Angew. Chemie, 98 (1986), 530-548, J. Chromat., 296 (1984), 107-114, Anal. Biochem., 137 (1984), 464-472).

A comparison of the hydrophobicity of substances suitable according to the invention is to be found in Sep. Sci. Technol., 14, 305-317 (1979). According to that, the hydrophobicity increases e.g. in the following series: α_2 -macroglobulin (M.W. 820,000)
bovine serum albumin/human serum albumin (M.W. $\sim$ 70,000)
egg albumin
 α_2 HS-glycoprotein (M.W. $\sim$ 49,000)
 β_{1c}/β_{1A} -globulin
immunoglobulin (M.W. $\sim$ 150,000)
transferrin (M.W. $\sim$ 90,000).

Thus, if an immunoglobulin is used as specifically bindable substance, then, for example, human serum albumin or α_2 HS-glycoprotein are not suitable as soluble proteins in the meaning of the invention without
5 further pre-treatment.

Both proteins must here be subjected not only to a hydrophobing but also to an increasing of the molecular weight. In the case of transferrin, in this case a cross-linking suffices, in the case of α_2 -
10 macroglobulin a hydrophobing.

Proteins which are suitable for the coupling with immunoglobulin as specifically bindable substance without pre-treatment are, for example, β -lipoproteins (M.W. about 3.2 mio.) and α_2 -lipoproteins (M.W. about
15 5 - 20 mio).

The hydrophobing can take place, for example, by the use of heat, treatment with acids, denaturing agents and/or chaotropic ions and/or by chemical coupling with a hydrophobic compound.

20 The increasing of the molecular weight can take place, for example, by the use of heat, treatment with acids, denaturing agents and/or chaotropic ions and/or by cross-linking with a bi- or polyfunctional protein reagent.

25 The treatment is carried out until a protein polymer is obtained with a molecular weight of 500,000 or more. It is especially preferred to use a protein polymer with a molecular weight of 500,000 to 20 mio.

30 When a cross-linking is also to take place, the hydrophobing can be carried out before, during or after the cross-linking but not in the presence of the specifically bindable substance.

For hydrophobing by heating, there are usually used temperatures of 40 to 95°C over a period of time
35 of 1 min to 10 hours, such as is described, for example,

in Biochem. Biophys. Acta, 624 (1980), 13-20.

As acids, there are used, for example, acetic acid, propionic acid, lactic acid or hydrochloric acid. Usual concentrations are 1 to 100 mMol/l, periods of
5 action of 10 min to 16 hours.

Suitable chaotropic ions are, for example, thiocyanates, iodides, fluorides, bromides, perchlorates and sulphates. Suitable denaturing agents are, for example, guanidine hydrochloride or urea.
10 Concentrations of 10 mMol/l to 6 mol/l are usually here used.

For the derivatisation with hydrophobic compounds, there are preferably used soluble fatty acids, lipoids in low or high molecular form, as well as synthetic
15 polymers, such as polypropylene glycol, or soluble copolymers of polystyrene. The derivatisation takes place according to methods known to the expert.

The cross-linking by way of bi- or polyfunctional compounds is carried out with per se known protein
20 binding reagents. These are compounds which contain at least two functional groups, which can be the same or different and which can react via these functional groups with functional groups of proteins. Compounds are preferably used which consist of an alkyl chain on
25 the ends of which are present succinimide, maleinimide and/or aldehyde groups.

The protein is cross-linked in per se known manner with the bi- or polyfunctional compounds in that the soluble protein and the bi- or polyfunctional
30 compound are reacted.

For the hydrophobing and/or cross-linking, there are preferably used proteins with a molecular weight of 10,000 to 700,000. Bovine serum albumin, lipase or immune γ -globulin are especially preferred.

The specifically bindable protein substance to be bound is then coupled to the protein in per se known manner. Suitable coupling methods are described e.g. in Ishikawa, J. Immunoassay, 4, 209-327 (1983).

5 Proteins such as for example antibodies, antibody fragments, antigens or haptens are suitable as specifically bindable substances.

The conjugate obtained of specifically bindable protein substance and protein is then adsorbed
10 adsorptively on the synthetic material surface serving as solid phase. The adsorptive binding to the solid phase takes place via strong and weak exchange actions, hydrophobic forces, dipole-dipole or ion-dipole interactions. As hydrophobic solid phases, there are suitable
15 able carrier materials with a surface tension which is smaller than the surface tension of the hydrophobic soluble protein, i.e. more hydrophobic than protein. Carrier materials with a surface tension of $< 40 \text{ erg/cm}^2$ are preferably used. Polystyrene, polymethacrylate,
20 Teflon, polyamide, co-polymers of styrene and acrylonitrile, glass and cellulose products, are especially suitable. They can be present in any desired form, e.g. as film, plate, powder, grains or fibre fleece, preferably as glass fibre fleece or fleece of
25 cellulose/cellulose ester fibre and polymer fibre.

Hydrophobed proteins display an especially good adsorptive binding. Due to the hydrophobing, intramolecular bridge bonds of the protein are possibly opened so that hydrophobic parts of the protein reach
30 the surface and there better adhere to the hydrophobic synthetic material surface than the hydrophilic parts which are particularly to be found on the surface in the non-hydrophobed protein.

The process according to the invention is suitable
35 able for the determination of a specifically bindable

substance. As substance pairs, one reaction partner of which is present bound to the solid phase, there are suitable, for example, antigen-antibody; hapten-antibody and other proteins capable of specific binding to one another, such as especially the system streptavidin or avidin and biotin, which is preferred.

Before the conjugate of protein and specifically bindable substance is adsorbed on to the hydrophobic solid phase, it is also possible to pre-treat the solid phase physically or chemically. Thus, for example, a synthetic resin surface can be pre-swollen or activated in some other per se known way.

The carrier material according to the invention for use in solid phase immunoassay is characterised in that it consists of a hydrophobic solid phase on which is adsorbed a protein with a molecular weight above about 500,000 to which is bound a specifically bindable substance.

This carrier material is outstandingly suitable for use in solid phase immunoassays since the specifically bindable substance adheres very well and is also not desorbed in the case of the addition of detergents.

The carrier material is present, for example, in the form of test tubes, microtitre plates or spheroids which are coated with a cross-linked protein to which is bound a specifically bindable substance.

On the solid phase is adsorbed a conjugate consisting of a cross-linked protein and a specifically bindable substance. The protein is preferably bovine serum albumin, lipase or an immune γ -globulin which has been cross-linked in the given way.

According to the invention, there is made available a process and a carrier material in order to fix a specifically bindable substance with good

adhesion and longlastingly to a hydrophobic solid phase. The adhesion could thus be improved to such an extent that even an addition of detergents does not lead to a dissolving off of the substance. The process according
5 to the invention is simple to carry out.

The invention is explained by Examples in conjunction with the drawing. In this are illustrated:

Fig. 1 a calibration curve for a TSH determination
with the use of non-crosslinked Fab <TSH>
10 (curve 1), crosslinked Fab <TSH> (curve 2),
conjugate of Fab <TSH>/ β -Gal (curve 3),
conjugate of Fab <TSH>/thermo-BSA (curve 4)
in Luran[®] test tubes;
Fig. 2 a calibration curve for a TSH determination
15 with the use of immobilised streptavidin and
biotinylated Ak <TSH>; non-crosslinked
streptavidin (curve 1), crosslinked streptavidin
(curve 2), streptavidin/ β -gal conjugate (curve 3),
streptavidin/BSA conjugate (curve 4),
20 streptavidin/thermo-BSA conjugate (curve 5),
streptavidin/thermo-BSA conjugate (curve 6).
Curves 1 to 5 in each case Luran[®] test tubes
as solid phase, curve 6 polystyrene test tubes
as solid phase.

25 Fig. 3 a calibration curve for a T3 test.

Fig. 4 a calibration curve for an HBs AG test.

Example 1.

Binding of Fab fragments of a monoclonal antibody
against TSH to polystyrene test tubes.

30 a) Preparation of Fab fragments (Fab <TSH>)

Monoclonal antibodies (MAB <TSH>) are obtained
by the method described by Galfre and Millstein (Meth.
in Enzymology, 73 (1981)). For the further purific-
ation, the ascites liquid is subjected to an ammonium
35 sulphate precipitation and to a passage over DEAE-
cellulose[®] .

A papain fission is subsequently carried out according to Biochem. J., 73 (1959) 119-126. The Fab fragments hereby formed are separated from the non-digested IgG molecules and the Fc fragments by means of gel filtration over Sephadex G100[®] and ion exchanger chromatography over DEAE-cellulose[®] according to Meth. in Enzymology, 73 (1981), 418-459.

b) Crosslinking of the Fab fragments without protein addition (comparison)

10 50 mg of Fab fragments are dissolved in 2 ml 0.05 mol/l potassium phosphate buffer (pH 7.5) and 0.4 ml disuccinimidyl suberate (manufacturer Pierce), dissolved in dioxane (7.4 mg/ml), is added thereto, with stirring. After 2 hours incubation at 25°C, the
15 reaction is broken off by the addition of 0.2 ml of 0.1 mol/l lysine hydrochloride. The reaction batch is diluted with 0.2 ml potassium phosphate buffer (v.supra) and centrifuged. The supernatant is desalinated over an Ultrogel[®] AcA 202 column (LKB, Grafelfing, Federal
20 Republic of Germany), whereby 11.3 ml are obtained with 45 mg of protein. A part of this preparation is fractionated on a Superose[®] -6-column (Deutsche Pharmacia GmbH) at a flowthrough rate of 0.5 ml/min in
25 0.05 mol/l potassium phosphate buffer pH 7.0 and the fractions with a molecular weight of about 500,000 to 5 mio further used.

c) Cross-linking of the Fab fragments with untreated γ -globulin (comparison)

30 Fab fragments and bovine γ -globulin (Serva, Heidelberg, Federal Republic of Germany) are mixed in a weight ratio of 1:1. The cross-linking is carried out as described under b).

d) Binding of Fab fragments to pre-cross-linked γ -globulin (according to the invention)

1.25 g γ -globulin are dissolved in 10 ml of

0.05 mol/l potassium phosphate buffer, pH 7.8, and centrifuged clear in a Sorvall cooled centrifuge for 10 min at 5000 r.p.m. 1.75 ml Disuccinimidyl suberate are added to this solution and diluted with 2.5 ml
5 water. After 4 hours stirring at 25°C, 10 ml of 0.1 mol/l lysine are added thereto and the pH value adjusted to 6.8 and centrifuged. The supernatant is separated on a preparative gel filtration column (TSK 3000, LKB Grafelfing, Federal Republic of Germany),
10 concentrated by ultrafiltration and stored at 4°C.

50 mg of this cross-linked γ -globulin are dissolved in 5 ml of 0.05 mol/l potassium phosphate buffer and the pH value adjusted to 9.5 by the addition of solid sodium carbonate. 50 mg N-acetylhomocysteine
15 thiolacetone (Serva, Heidelberg, Federal Republic of Germany) are then added thereto and stirred for 5 hours at 25°C, with nitrogen gassing. The batch is subsequently desalinated over an Ultragel[®] AcA 202 column in a buffer of 0.1 mol/l potassium phosphate (pH 7.0),
20 0.001 mol/l magnesium chloride and 0.05 mol/l sodium chloride.

Fab fragments prepared according to a) (10 mg in 1 ml of 0.01 mol/l potassium phosphate buffer pH 7.0) are activated with 0.002 ml maleinimidohexanoyl-
25 N-hydroxysuccinimide ester (Boehringer Mannheim GmbH) in DMSO (33 mg/ml) for 2 hours at 25°C, subsequently centrifuged and desalinated over an AcA 202 column.

These fragments are subsequently combined with the γ -globulin (weight ratio of the proteins 1:1) and
30 incubated for 1 hour at 25°C and at pH 7.0. Subsequently, it is dialysed against desalinated water overnight at 4°C and at a protein concentration of 2.5 mg/ml.

This conjugate can be used directly for the
35 adsorption on to a solid phase.

e) Preparation of Fab fragments coupled to thermo-BSA
(bovine serum albumin; according to the invention)

Preparation of thermo-BSA (bovine serum albumin):

1 g BSA-I are dissolved in 100 ml of 50 mMol/l
5 potassium phosphate (pH 7.0), heated to 70°C and kept
at this temperature for 4 hours, with gentle stirring.
The solution is cooled, filtered and adjusted in an
ultrafiltration cell (exclusion limit: 30,000 Dalton)
to a concentration of 50 mg/ml. Subsequently, it is
10 dialysed against a 30 fold volume of double distilled
H₂O and subsequently lyophilised. The product has a
molecular weight of about 700,000.

Before coupling to the Fab fragments, the thermo-
BSA is activated. For this purpose, 68 mg thermo-BSA
15 are dissolved in 2 ml 0.1 mol/l potassium phosphate
(pH 7.8) and slowly mixed with a solution of 3.8 mg
S-acetylmercaptosuccinic acid anhydride (SAMSA). After
a reaction time of 3 hours, it is dialysed against
2 l of 50 mMol/l potassium phosphate (pH 6.5). This
20 thermo-BSA is incubated for 1 hour at 25°C and pH 7.0
with the Fab fragments activated according to d) in a
weight ratio of 1:1, subsequently dialysed against
desalinated water overnight at 4°C and at a protein
concentration of 2.5 mg/ml.

25 This product is used directly for the coating.

f) Preparation of Fab fragments coupled to
β-galactosidase (according to the invention)

Fab fragments are activated as described in d)
and coupled to the SH groups of β-galactosidase accord-
30 ing to J. Immunoassay, 4 (1983), 209-327. The β-
galactosidase used hereby has a molecular weight of
500,000 to 2 mio. For another experiment, cross-linked
β-galactosidase (M.W. ~ 5 mio) was used.

g) Loading of polystyrene test tubes with Fab
35 fragments or their conjugates

50 mg of a lyophilisate of the Fab fragment or of the conjugate are dissolved in 10 ml double distilled water. 1 ml of this solution is diluted in 1000 ml of a loading buffer of 5.25 g/l sodium dihydrogen phosphate and 1 g/l sodium azide and stirred for 30 min at room temperature.

Test tubes of polystyrene or Luran[®] (manufacturer BASF) are each filled with 1.5 ml of the solution and loaded overnight (about 22 hours). Thereafter, the test tubes are completely emptied and the function test described in the following is carried out.

h) Function test via TSH determination

The polystyrene and Luran[®] test tubes coated according to g) are used in a TSH determination reagent analogously to TSH-Enzymun[®] test (Boehringer Mannheim GmbH, order No. 736 082) and a calibration curve measured according to the test procedure. There are hereby obtained the measurement values shown in Table 1 and Fig. 1, respectively.

It is to be seen (Fig. 1) that with Fab fragments which have been immobilised without protein addition, only a very flat calibration can be obtained (curves 1 and 2). By coupling to proteins with a molecular weight below 500,000 and low hydrophobicity, the calibration curve (curve 3) is steeper. However, satisfactory results still cannot be achieved. On the other hand, with the conjugates prepared according to the invention, there can be achieved a sufficiently steep calibration curve (curve 4).

Table 1
Standard values for various Fab <TSH. conjugates

conjugate *	hydrophobicity of the protein **	M.W. of the protein	measurement signal of TSH standard in mE/ml	
			0.1 μ U TSH ¹⁾	12/5 μ U TSH ²⁾
Fab <TSH>	-	-	102	107
cross-linked Fab <TSH> without protein (Example 1b)	-	-	126	134
Fab <TSH>/ β -Gal (Example 1f)	39.6	500,000	110	260
cross-linked Fab <TSH>/ β -gal (Example 1f)	40.2	5 mio	110	260
Fab <TSH>/ γ - globulin (Example 1c)	32.0	150,000	117	253
Fab <TSH>/ γ - globulin (Example 1d)	not elutable	> 5 mio	75	480

* total amount of protein in the case of adsorption: 1.5 ml with 5 μ g/ml of protein per test tube

** Tp [min], cf. Example 3

1) lowest value of a measurement curve (should be as low as possible)

2) highest value of a measurement curve (should be as high as possible)

Example 2.

Immobilisation of streptavidin

a) Preparation of cross-linked streptavidin

Streptavidin (manufacturer: Boehringer Mannheim GmbH) is cross-linked analogously to Example 1b.

b) Preparation of streptavidin bound to BSA or to β -galactosidase

The binding to non-cross-linked BSA or to β -galactosidase takes place analogously to Example 1f.

c) Preparation of streptavidin coupled to thermo-BSA.

Activation of streptavidin:

60 mg Streptavidin are dissolved in 6 ml of 50 mMol/l potassium phosphate/100 mMol/l sodium chloride (pH 7.0) and cold stored at 4°C. 6.16 mg maleinimido-hexanoyl-N-hydroxysuccinimide ester are dissolved in 620 μ l dioxane and stirred into the streptavidin solution. After a reaction time of 2 hours at 4°C, it is dialysed twice against 1 l of 50 mMol/l potassium phosphate/100 mMol/l sodium chloride (pH 5) at 4°C.

Preparation of a conjugate of streptavidin and thermo-BSA:

66 mg Streptavidin are dissolved in 10 ml of 50 mMol/l potassium phosphate pH 5.0 and 100 mMol/l sodium chloride and 72 mg of activated thermo-BSA-SAMBA (preparation according to Example 1e)) in 5 ml of 50 mMol/l potassium phosphate/100 mMol/l sodium chloride pH 6.5 are added thereto. After mixing, 50 μ l of 1 mol/l hydroxylamine pH 7.0 are added thereto in order to stop the reaction. After 3 hours, the reaction product is purified via gel chromatography (Superose[®] 6, 50 mMol/l potassium phosphate/100 mMol/l sodium chloride pH 7.5). There is obtained a conjugate with a molecular weight between 1 and 5 mio.

d) Loading of polystyrene and Luran[®] test tubes
with streptavidin or streptavidin conjugate

The loading takes place as described in Example 1g).

5 e) Measurement of standard values via a TSH
determination

The test tubes loaded according to d) are used in a TSH Enzymun[®] test reagent (4 fold conjugate activity). However, in variation of the there-described procedure,
10 instead of the immobilised antibody, there is used a biotinylated MAB <TSH>. The preparation of this biotinylated MAB takes place according to J.A.C.S., 100 (1978), 3585-3590. The antibody is used in the test in a concentration of 400 ng per test tube, together with
15 the other reagents.

Fig. 2 shows the results. It is to be seen that, with increasing molecular weight and increasing hydrophobicity, the gradient of the calibration curve and thus of the achievable exactitude increases.

20 Example 3.

A T3 test was carried out with the test tubes loaded according to the invention. For this purpose, 200 µl of sample or of standard solution were pre-incubated together with 500 µl of a polyclonal antibody
25 conjugate against T3, which was labelled with POD, in a test tube which was coated with streptavidin coupled to thermo-BSA. After 5 minutes, 400 ng of polymerised T3 biotinylated in known manner were incubated for 30 minutes in 500 µl of buffer. As buffer, there was
30 used a solution of sodium hydrogen phosphate with a pH of 8.65 which contains 0.20% BSA and 0.04% 8-anilino-1-naphthalenesulphonic acid (ANA). After the incubation, it was washed three times and subsequently 1 ml ABTS substrate solution added thereto. After further 30
35 minutes incubation, it was then measured at 405 nm in a

photometer. The measurement values obtained are to be seen from the curve shown in Fig. 3.

Example 4.

5 An HBaAg test was carried out with test tubes coated according to the invention. For this purpose, in a test tube coated according to Example 1g), there were simultaneously dissolved 400 ng biotinylated monoclonal antibody against HBs-antigen and 50 mU of the same antibody which were labelled with POD and 1 ml
10 of buffer of the same composition as described in Example 3 added thereto, together with 200 µl of standard solution. As standard solution, there was added, on the one hand, purified HBsAg sub-type ay and, on the other hand, purified HBsAg sub-type ad. It was
15 then incubated for 4 hours at room temperature. After washing the test tubes three times, 1 ml ABTS substrate solution was added thereto. After 20 minutes, the reaction was substantially ended and the extinction was measured at 405 nm in a photometer. The measurement
20 values are to be seen from Fig. 4, whereby the unbroken curve gives the values for HBsAg sub-type ad and the broken curve the values for HBsAg sub-type ay.

Example 5.

25 The dissolving off of the test tubes coated according to the invention was compared with test tubes coated according to known processes. For this purpose, on the one hand, test tubes were loaded with 1.5 ml of a streptavidin-thermo-BSA solution (4 µg/ml) in 40 mM sodium hydrogen phosphate buffer, pH 7.4, at 20°C for
30 18 to 24 hours. After sucking out of the test tubes, there took place an after-loading with 1.8 ml of 2% saccharose solution which contained 0.9% sodium chloride and 0.3% BSA. The after-loading took place for 30 minutes at 20°C. Subsequently, the test tubes were
35 dried for 24 hours at 20°C and 40% relative humidity.

These test tubes are ready for use for the carrying out of tests. Furthermore, test tubes were loaded in precise known manner with pure streptavidin. The dissolving off behaviour in the case of the action of detergents was tested with these test tubes. The results are to be seen from the following Table.

Table 2

test tube		% dissolving off	% dissolving off	% dissolving off	% dissolving off
loading	material	1% Pluronic F 68 (30 min, RT)	0.5% Triton (30 min,RT)	function test (30 min,20°C)	function test (30 min, 30°C)
Streptavidin (comparison)	Luran	13.9	42	17.8	28
	polystyrene	11	25	9.6	11.6
thermo-BSA- streptavidin (invention)	Luran	2.1	8	3.1	11.6
	polystyrene	1.5	2.1	1.6	1.7

The determination of the percentage dissolving off took place according to the methods known to the expert, e.g. via a ^{125}I -labelling of streptavidin and streptavidin-thermo-BSA or an enzymatic determination.

5 For the enzymatic determination of the percentage dissolving off, the loaded test tubes were incubated under the conditions according to Table II with 50 mMol/l potassium phosphate buffer, pH 7.0, to which had been added the detergent according to Table II.

10 Subsequently, it is incubated for 1 hour at room temperature, washed with the above-mentioned buffer and incubated for 1 hr. at room temperature with a conjugate of biotin-POD (200 mU/ml POD activity), washed and 2 ml ABTS $\text{\textcircled{R}}$ solution (Example 7) added thereto. After
15 1 hr. substrate reaction, the extinction is determined at 405 nm and the percentage dissolving off of streptavidin or streptavidin-thermo-BSA determined herefrom via a standard calibration curve.

Example 6.

20 Determination of the hydrophobicity of proteins with hydrophobic interaction chromatography (HIC)

The hydrophobicity of various compounds was investigated with a liquid chromatograph (Hewlett Packard 1090 LUSI). The pre-column was a BioRad
25 Biogel $\text{\textcircled{R}}$ TSK-phenyl-5PW column (L 5 mm x ID 4.6 mm), column: BioRad-Biogel $\text{\textcircled{R}}$ TSK-phenyl-5PW (L 75 mm x ID 7.5 mm, 10 μm 1000 Å). As detector, there was used a Hitachi F 1000 fluorimeter.

Eluents/gradient (Table 3).

30 a) 1.5 mol/l $(\text{NH}_4)_2\text{SO}_4$ solution in 1/100 mol/l KH_2PO_4 buffer, pH 6.8

b) 1/100 mol/l KH_2PO_4 buffer, pH 6.8

Table 3

	A%	B%	t/min.
	100	0	0
	100	0	5
5	0	100	30 flow = 0.5 min
	0	100	5
	100	0	5
	100	0	5

Working temperature: cold chamber +7°C.

10 Sample preparation:

The samples were used undiluted. The sample volume amounted to 10 μ l.

The compounds to be investigated were dissolved at a concentration of 0.2 to 1.4 mg/ml in 10 mMol/l
15 potassium phosphate buffer pH 6.8.

In Table 4 are summarised the retention times for various proteins and specifically bindable substances. The longer the retention time, the greater the hydrophobicity.

20 Table 4

	protein	retention time t_p (min.)
	Fab TSH	41.5
	BSA	23.2
25	γ -globulin	32.0
	β -galactosidase	39.6
	cross-linked β -galactosidase	40.2
	streptavidin	38.3
	thermo-BSA (Example 1e)	not elutable
30	γ -globulin, cross-linked (Example 1a)	not elutable

The proteins not elutable under these conditions are especially suitable and are preferably used. Thermo-BSA is quite especially preferred.

Example 7.

5 Adsorption of thermo-BSA-streptavidin on glass fibre fleece

A glass fibre fleece (6 x 6 mm) is impregnated in the absorption volume (about 30 µl) with a solution of 30 µg/ml thermo-BSA-streptavidin (preparation
10 Example 2c) in 50 mMol/l potassium phosphate buffer, pH 7.0, and dried in a circulating cabinet at 50°C.

For the determination of the biotin binding capacity, the strip is impregnated with 30 µl of a reagent consisting of conjugate of peroxidase (POD) and
15 biotin with a POD activity of 50 mU/ml, biotin standard with the concentrations 0.5, 10, 20, 30, 40, 50, 100, 200, 1000 ng/ml biotin, and incubated for 2 minutes. Subsequently, it is washed once with an excess of 50 mMol/l potassium phosphate buffer, pH 7.0, 2% Tween[®]
20 20 and subsequently introduced into 2 ml ABTS solution.

100 mMol/l citrate buffer, pH 4.4

3.2 mMol/l perborate

1.9 mol/l ABTS[®] (2,2'-azino-di-[3-ethyl-
benzthiazoline-sulphonic acid (6)]
25 diammonium salt)

and kept in movement for 15 minutes. After 1 hour substrate reaction, the extinction is determined at 405 nm and the biotin binding capacity determined herefrom via the half maximum signal decrease.

Patent Claims

1. Process for the preparation of a specifically bindable protein substance bound to an insoluble carrier material, especially for use in a heterogeneous analysis process, according to the immunoassay principle, characterised in that one couples a soluble protein with a molecular weight above about 500,000, which is more hydrophobic than the specifically bindable substance, to the specifically bindable substance and then adsorbs the conjugate of reaction component and protein on a hydrophobic solid phase.
2. Process according to claim 1, characterised in that, as soluble protein, one uses a protein with a molecular weight of 500,000 to 20 mio.
3. Process according to claim 2, characterised in that one prepares the soluble protein from a protein with a molecular weight of 10,000 to 70,000 by increasing the molecular weight to above 500,000 to 20 mio.
4. Process according to one of claims 1 to 3, characterised in that, before coupling, one cross-links the protein with a bi- or polyfunctional protein reagent until the desired molecular weight is achieved.
5. Process according to one of the preceding claims, characterised in that one uses a hydrophobed protein.
6. Process according to claim 5, characterised in that, before coupling, the protein is hydrophobed by the use of heat, treatment with acids, denaturing agents or chaotropic ions and/or by chemical coupling with a hydrophobic compound.
7. Process according to claim 4, characterised in that one uses disuccinimidyl suberate as bifunctional compound.



8. Process according to one of claims 3 to 7, characterised in that one uses bovine serum albumin, lipase and/or immune γ -globulins as protein.
- 5 9. Carrier material for use in solid phase immuno-assays obtainable according to one of claims 1 to 8, characterised in that it consists of a hydrophobic solid phase on which is adsorbed a conjugate of a hydrophobic protein with a molecular weight above about 500,000 and of a specifically bindable protein
- 10 substance.
10. Carrier material according to claim 9, characterised in that the hydrophobic solid phase consists of polystyrene, polymethacrylate, polyamide, Teflon^(R) or a co-polymer of styrene and acrylonitrile.
- 15 11. Carrier material according to claim 9 or 10, characterised in that the protein is hydrophobed bovine serum albumin (thermo-BSA), hydrophobed lipase or hydrophobed immune γ -globulin.
- 20 12. Carrier material according to one of claims 9 to 11, characterised in that the specifically bindable substance is an antigen or an antibody.
13. Carrier material according to one of claims 9 to 11, characterised in that the specifically bindable substance is streptavidin or avidin.
- 25 14. Carrier material according to one of claims 9 to 13, characterised in that the carrier is a glass fibre fleece or a fleece of cellulose/cellulose ester fibres and polymer fibres.

