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Enzymic Preparation of Protein G–peroxidase Conjugates Catalysed by Transglutaminase[1]

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Transglutaminases (TGases, EC 2.3.2.13) have proved to be valuable enzymes for site-directed protein coupling *via* N^{ε}-(γ-L-glutamyl)lysine bonds. Their use in conjugate synthesis would overcome many problems caused by chemical reagents. In this approach, we show for the first time that two proteins with different functionalities, namely soybean peroxidase and protein G, can be cross-linked by bacterial TGase with retention of their activities. Soybean peroxidase and protein G were chosen for the enzymic preparation of a bifunctional conjugate among a series of other TGase substrates detected by enzymic incorporation of small fluorescent or biotinylated molecules. The highest yields of conjugate were obtained with a 15-fold excess of peroxidase in phosphate buffer, pH 7.0. Size exclusion chromatography enabled both purification of the conjugates and recovery of the starting materials. Analysis of bifunctionality reveal-ed the coupling of protein G with an average of three peroxidase molecules.

Key words: enzymic conjugate preparation, enzymic protein coupling, enzymic protein labeling, protein G–peroxidase conjugates, transglutaminase.

Conjugates have been shown to be valuable tools in immunochemical analysis. Coupling products containing an enzyme are frequently used, especially if the handling of radioactive markers is to be avoided. Stable intermolecular cross-links between at least two compounds with different activities are usually generated by chemical reagents often resulting in low conjugate yields. This may be due to the uncontrolled course of the coupling reaction, and gentler, more precisely directed procedures would be desirable.

Transglutaminase (TGase) catalyses the intermolecular cross-linking of proteins by acyl transfer of protein-bound glutamine to lysine (*1*). Formation of the resulting isopeptide bond is highly specific and depends on the proteins involved and the cross-linking enzyme. Thus, the enzymic reaction fails if the amino acids concerned, especially glutamine, are not accessible to TGase (*2*). Additionally, the coupling enzymes may differ in their specificities for various glutamine residues. For instance, human blood factor $XIII_a$ modifies glutamine-167 of β-casein while tissue TGase prefers glutamine-79 (*3*, *4*). This high specificity of mammalian enzymes may be one reason for the low number of proteins that have hitherto been identified as substrates of TGase, in most cases associated with distinct physiological functions. Important proteins used for the preparation of conjugates are not found among them.

Bacterial TGase from *Streptoverticillium* sp. has been used in many procedures for modifying food proteins (*5*). It has also been shown that the inclusion of macromolecules into protein matrices cross-linked by TGase may be a useful method for the immobilization of bioactive proteins (*6*, *7*). However, participation of the enzymes in the TGase reaction was not evident. It must be added that the specificity of the bacterial enzyme is completely unknown. Only a few attempts have been made to characterize bacterial TGase using synthetic peptide derivatives (*8*, *9*).

For the enzymic synthesis of conjugates, it was therefore important to detect functional proteins bearing accessible glutamine and lysine residues. This was achieved by TGase-catalyzed labeling of enzymes and other bioactive proteins using fluorescent and biotinylated compounds (*9*–*11*). The results obtained suggested protein G and soybean peroxidase as suitable proteins for preparing bifunctional conjugates.

MATERIALS AND METHODS

Peroxidases (donor: hydrogen-peroxide oxidoreductase; EC 1.11.1.7) from bovine milk, horseradish, soybean and *Arthromyces ramosus*, β-galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) recombinant from *Aspergillus oryzae* and an overproducing strain of *Escherichia coli*, alkaline phosphatase (orthophosphoric-monoester phosphohydrolase [alkaline optimum]; EC 3.1.3.1) from bovine intestinal mucosa, protein A from *Staphylococcus aureus*, recombinant protein G from *Streptococcus* sp. expressed in *Escherichia coli*, avidin from chicken egg white, streptavi-

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Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); BrClIndP, 5-bromo-4-chloro-3-indolylphosphate; CBZ-Gln-Gly-DC, 1-*N*-(carbobenzoxy-L-glutaminylglycyl)-5-*N*-(5'-*N'*,*N'*-dimethylaminonaphthalenesulfonyl)diamidopentane; MBC, monobiotinylcadaverine; MDC, monodansylcadaverine; NBT, nitro blue tetrazolium; NC, nitrocellulose; TGase, transglutaminase.

din from *Streptomyces avidinii*, bovine serum albumin, anti-chicken (rabbit) immunoglobulin-alkaline phosphatase conjugates, streptavidin-alkaline phosphatase conjugates, anti-rabbit immunoglobulin from goat, 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), nitro blue tetrazolium (NBT), 4-chloro-1-naphthol, *p*-nitrophenyl phosphate, and monodansylcadaverine (MDC) were obtained from Sigma (Deisenhofen, Germany). 5-Bromo-4-chloro-3-indolylphosphate (BrClInd*P*)/NBT tablets were from Schleicher&Schuell (Dassel, Germany), monobiotinylcadaverine (MBC) and a bicinchoninic acid protein assay was from Pierce (Rockford, USA). Protein analysis was carried out according to the manufacturer's protocol. Protein standards for polyacrylamide gel electrophoresis, size exclusion chromatography, and isoelectric focussing were from Sigma, Bio-Rad (Munich, Germany) and Serva (Heidelberg, Germany), respectively. Bacterial transglutaminase (protein-glutamine: amine γ-glutamyltransferase; EC 2.3.2.13), prepared as described by Gerber *et al.* (*12*), was used with an activity of 16 U/mg (hydroxylaminolysis reaction), see Grossowicz *et al.* (*13*). All other chemicals were supplied in the finest quality available by Applichem (Darmstadt, Germany), Bio-Rad, Fluka (Buchs, Switzerland), Merck (Darmstadt, Gemany), Serva, and Sigma.

Labeling of Bioactive Proteins—Protein-labeling was carried out in 0.5 M Tris-HCl, pH 7.5, or 0.1 M phosphate buffer, pH 7.3, at 37°C for 1 h by bacterial TGase catalyzed incorporation of MDC **1**, MBC **2**, or 1-*N*-(carbobenzoxy-L-glutaminylglycyl)-5-*N*-(5'-*N'*,*N'*-dimethylaminonaphthalenesulfonyl)diamidopentane (CBZ-Gln-Gly-DC) **3** (Fig. 1) according to the methods of Lorand *et al.* (*10*), Singh *et al.* (*11*), and Pasternack *et al.* (*9*) as listed in Table I. The reaction was terminated by heating (60°C) in application buffer containing 20% (v/v) glycerol, 20% (w/v) urea, and 4% (w/v) SDS, and the products were analysed by SDS-PAGE as described below.

Protein Cross-Linking—Protein G or soybean peroxidase were incubated with bacterial TGase (molar ratio of 10:1) in 0.05 M phosphate buffer, pH 7.5, at 37°C for 24 h. Subsequently, the products were analysed by PAGE with silver staining.

Preparation of Conjugates—In order to find the optimal

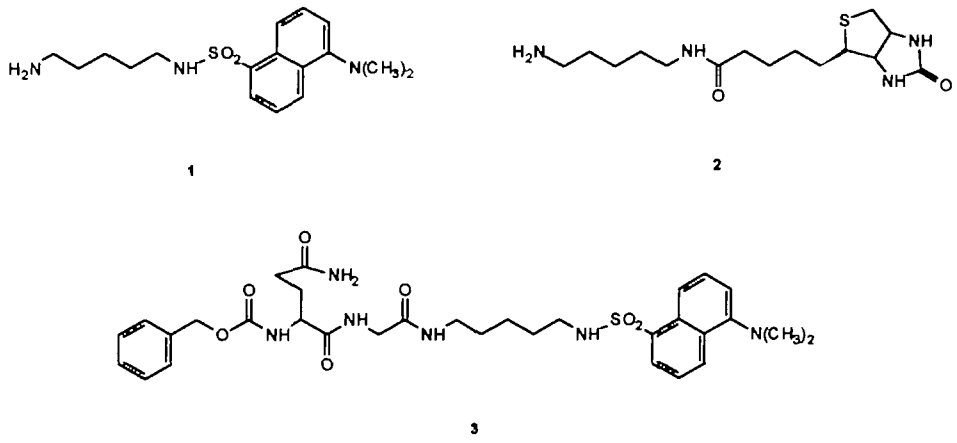


Fig. 1. **Compounds for identification of TGase substrates.**

TABLE I. **Composition of the reaction mixtures used for the enzymic labeling of bioactive proteins catalysed by bacterial transglutaminase.**

Proteins	Proteins (nmol/ml)	MBC (nmol/ml)	MDC (nmol/ml)	CBZ-Gln-Gly-DC (nmol/ml)	TGase (nmol/ml)	Buffer	Final volume (μl)	Application buffer (μl)	Applicated protein (μg)
Protein A from *Staphylococcus aureus*	15.0		4,500	1,500	0.3	Phosphate	70	80	5
	4.8	500			0.05	Tris	250	750	5
rec. protein G	15.0		4,900	1,500	0.3	Phosphate	70	50	5
	6.7	500			0.05	Tris	250	750	5
Avidin from chicken egg white	3.79		2,750	500	0.45	Tris	40	10	5
Streptavidin from *Streptomyces avidinii*	4.17		2,750	500	0.45	Tris	40	10	5
Alkaline phosphatase from bovine intestinal mucosa	1.4	500			0.05	Tris	250	750	12
	4.8		2,840	1,000	0.19	Phosphate	30	20	12
Peroxidases from bovine milk	9.5		2,200	800	0.15	Phosphate	30	20	12
	2.9	500			0.05	Tris	250	750	12
Horseradish	4.4	500			0.05	Tris	250	750	12
Soybean	15.0		4,500	1,500	0.3	Phosphate	70	30	12
	5.3	500			0.05	Tris	250	750	12
Arthromyces ramosus	15.0		4,500	1,500	0.03	Phosphate	70	30	12
	5.3	500			0.05	Tris	250	750	12
β-Galactosidase from *Escherichia coli*	0.4	500			0.05	Tris	250	750	12

coupling parameters, protein G (2.7 μM) was incubated with soybean peroxidase (molar ratios in the range of 1:1 to 1:50) and bacterial TGase (molar ratios to protein G in the range of 1:0.03 to 1:0.8) in different buffers, pH 6.0–8.5, for 3–24 h. The enzyme activity was measured at 405 nm after specific binding onto immunoglobulin G as outlined below. Conjugate mixtures obtained under the optimized conditions were purified by size exclusion chromatography.

Size Exclusion Chromatography—Retention volumes and apparent molecular weights M_r of protein G and peroxidase were determined using a 60 cm × 1.6 cm Superdex 200 column (Pharmacia) and a calibration curve obtained with a protein standard mixture from Sigma. Elution was carried out with 50 mM phosphate buffer, pH 7.0, containing 150 mM NaCl at room temperature. Cross-linked protein or conjugate mixtures (1 ml) were separated from starting materials under the same conditions.

Electrophoresis, Isoelectric Focusing, and Blotting—All commercially supplied proteins, the dansylated and biotinylated samples, and the conjugates purified by size exclusion chromatography were analyzed by PAGE (Mini-Protean II, Bio-Rad, Munich, Germany; 200 V, about 50 min, without cooling) using 10% or 12.5% separating gels (55 mm × 85 mm × 0.7 mm) and stacking gels (10 mm × 85 mm × 0.7 mm) of 5% polyacrylamide according to the method of Laemmli (*14*). After separation was complete, the gels were silver stained as described by Blum *et al.* (*15*) whereas, in the case of dansylated samples, prior visualization was achieved by U.V. irradiation at 366 nm. Biotinylated proteins were blotted onto nitrocellulose (NC) membranes (Schleicher&Schuell) in a Trans-Blot apparatus (Bio-Rad; 20 V, 45 min) according to Towbin *et al.* (*16*) and stained using streptavidin–alkaline phosphatase conjugates as described by Singh *et al.* (*11*). Starting materials and conjugate fractions (desalted using Bio-Rad PD 10 tubes) were also analysed by isoelectric focusing (Multiphor II, Pharmacia; 4°C; 200–300 V, 30 min for pre-focusing; 300–1500 V and 2.5 h for separating) using Servalyt gradient gels (Precotes 3–10 and Servalyt PreNets 3–10, Serva). The proteins were silver-stained or blotted as described above.

Detection of Blotted Protein G—The NC membrane loaded with protein G or a conjugate sample was washed three-times with 10 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl and 0.05% (v/v) Tween 20, 5 min each, and blocked with NaCl/Tris/Tween containing non-fat milk (room temperature, 1 h). After further washings with NaCl/Tris/Tween, rabbit antibody-alkaline phosphatase conjugates (1:15,000 diluted with NaCl/Tris/Tween) were added for 1 h followed by washing with NaCl/Tris/Tween (threefold, 5 min each) and 0.1 M Tris-HCl, pH 9.5, containing 0.1 M NaCl and 5 mM $MgCl_2$ (1 min). Coloring up to the desired intensity was obtained by incubation with BrClInd*P*/NBT (1 tablet in 30 ml water). The reaction was terminated by washing with 20 mM Tris-HCl, pH 8.0, containing 5 mM EDTA.

Detection of Blotted Peroxidase—Peroxidase or the peroxidase conjugates blotted on NC membranes were washed as described above and visualised by incubation with 4-chloro-1-naphthol (0.05% w/v) and 30% hydrogen peroxide (0.05% v/v) in 10 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 0.05% (v/v) Tween 20, and 17% (v/v) methanol. The color reaction was terminated by washing with water.

Quantitative Measurements of Peroxidase and Protein G Activities—Microtiter plates were coated with 100 μl of samples containing peroxidase (0.5–2 mg/ml), protein G (0.25–2.5 mg/ml), or protein G–peroxidase conjugates in appropriate dilutions for 1 h at room temperature. After the unbound protein was removed, the wells were blocked with 200 μl BSA [1% (w/v) in coating buffer, pH 8.3, containing 40 mM Tris-HCl and 150 mM NaCl] for 30 min, and washed three times (each 10 min) with 200 μl of 0.1 M phosphate buffer, pH 5.0 (peroxidase or peroxidase conjugates) or 0.9 M diethanolamine buffer containing 0.5 M $MgCl_2$ (protein G or protein G conjugate). Peroxidase activity was measured by adding 100 μl of 9.1 mM ABTS and 0.3% (v/v) H_2O_2 in phosphate buffer, pH 5.0, and reading the absorbance (405 nm) at 2 min intervals for 60 min using a Miniscan 340 reader manufactured by Asys Hitech (Eugendorf, Austria) and an evaluation program supplied by Mikrotek (Overath, Germany). For measuring protein G, each well (coated with protein G or protein G conjugates) was incubated with 100 μl of 1:5,000 diluted rabbit antibodies conjugated with alkaline phosphatase for 60 min and washed three times (each 10 min) with 1.6 M diethanolamine containing 0.5 M $MgCl_2$. After the liquid was discarded, 100 μl of 7.6 mM *p*-nitrophenyl phosphate in 1.6 M diethanolamine/$MgCl_2$ was added, and the absorbance was measured at 405 nm as described above.

Determination of Conjugates—Microtiter plates were coated and blocked with 100 μl rabbit IgG (0.1 mg/ml in 40 mM Tris-HCl, pH 8.3, containing 150 mM NaCl) for 60 min and 200 μl 1% BSA in Tris/NaCl for 30 min, respectively. After washing three times with 200 μl Tris/NaCl (each 10 min), the wells were incubated with 100 μl of protein G–peroxidase conjugate in an appropriate dilution for 1 h. Peroxidase activity was analysed in the same way as outlined above. Incubation mixtures without bacterial TGase containing only protein G and peroxidase provided the control values.

RESULTS AND DISCUSSION

Protein Screening—Many commercially available protein preparations were examined for purity by PAG electrophoresis. Further characterization was undertaken if the protein of interest could be clearly identified. That was only the case when the preparations were nearly homogeneous. The remaining products, among them different proteins with functionalities important in conjugate synthesis (Table II), were proved to be TGase substrates by enzymic incorporation of the fluorescent compounds MDC and CBZ-Gln-Gly-DC (*9*, *10*). To ensure that labeled proteins with weak fluorescence are actually glutamine substrates, more sensitive staining with MBC and streptavidin-alkaline phosphatase conjugates according to the method of Singh *et al.* (*11*) was also carried out.

As can be seen from Table II, more than half of the proteins studied were labeled, but only a few showed high fluorescence intensities, namely protein A, protein G, and avidin. Protein G and avidin may serve only as lysine donor compounds even if a small but significant amount of labeled protein G could be detected by the incorporation of MBC. Protein A has both accessible glutamine and lysine residues as can be seen by the successful coupling of protein A with either MDC or CBZ-Gln-Gly-DC. The fluores-

cence intensities obtained suggest that protein A is the strongest glutamine and lysine donor among the proteins in Table II.

The enzymes found to be substrates of TGase also have reactive glutamine and lysine side-chains, but the emission intensities of the labeled proteins were comparably low. Soybean peroxidase, bovine alkaline phosphatase and β-galactosidase from *E. coli* seemed to be the most suitable glutamine donors for the preparation of conjugates.

Selection of Suitable Proteins for Conjugation—In most cases, a conjugate for enzyme immuno assays is composed of a binding constituent, often an immunoglobulin directed against the first antibody, and an enzyme that catalyses the release of a colored compound. Conjugation is usually carried out by uncontrolled chemical cross-linking reactions. In contrast, enzymic cross-linking catalysed by TGase is specific, occurring solely between a glutamine and a lysine donor molecule. In an ideal case, an exclusive glutamine donor with one reactive side-chain may be coupled to a 1:1 conjugate with a protein bearing only one accessible lysine residue (Fig. 2, path 1), but, in general, TGase substrates are provided with both reactive amino acids (see Table II). Therefore, side-products without bifunctionality and high molecular size protein aggregates are to be expected (for examples see Fig. 2, paths 2–4).

Our screening shows that antibody- or biotin-binding proteins are excellent substrates of TGase (Table II). The labeling of rabbit immunoglobulin G did not succeed (not shown). Therefore, the antibody-binding proteins, protein A and protein G, were the first choice for preparing conjugates with a broad application spectrum. The properties of protein A and protein G are similar in many respects but a lack of reactive glutamines should prevent polymerisation of the latter.

Correspondingly, treatment of protein A and protein G with bacterial TGase, confirmed these considerations. When protein A was cross-linked by bacterial TGase, oligomers as well as highly polymerized aggregates not able to penetrate the separation and stacking gels were observed (not shown). In contrast, treatment of protein G with bacterial TGase followed by PAGE gave no substantial proof that considerable levels of polymerisation had occurred. Small polymeric bands at the top of the stacking and separation gels may be due to cross-linked protein impurities (Fig. 3, lanes 1 and 2). This estimation was confirmed by an additional band at about 25 kDa. The exclusive lysine donor protein G is obviously predestined for site-directed conjugate preparation catalysed by TGase.

The enzymes found to be TGase substrates represent the amplifying part of a conjugate. Low fluorescence intensities of the labeled proteins suggest that the number of reactive amino acids is rather limited. Nevertheless, soybean peroxidase, which was chosen as a model system for the enzymic coupling to protein G, could be cross-linked by TGase (Fig. 3, lane 4).

According to the labeling and cross-linking experiments,

TABLE II. **Visually estimated fluorescence and color intensities of bioactive proteins after enzymic labeling and staining using different marker molecules.**

Proteins	Glutamine donors identified by incorporation of		Lysine donors identified by incorporation of CBZ-Gln-Gly-DC (*9*)
	MDC (*10*)	MBC (*11*)	
Protein A from *Staphylococcus aureus*	High	High	High
rec. protein G	None	Low	High
Avidin from chicken egg white	None	n.d.	High
Streptavidin from *Streptomyces avidinii*	Low	n.d.	Medium
Alkaline phosphatase from bovine intestinal mucosa	Medium	Medium	Medium
Peroxidases from bovine milk	Low	Medium	Low
Horseradish	None	None	None
Soybean	Medium	Medium	Low
Arthromyces ramosus	Low	Low	Low
β-Galactosidase from *Escherichia coli*	n.d.	Medium	n.d.

n.d., not determined.

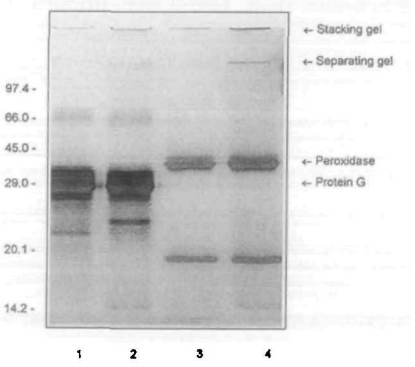


Fig. 3. **SDS–polyacrylamide gel electrophoresis of protein G and soybean peroxidase polymerised by TGase.** Mixtures containing protein G and bacterial transglutaminase or peroxidase and TGase were incubated in 0.1 M phosphate buffer, pH 7.5, at 37°C for 24 h. Lane 1, protein G; lane 2, protein G and bacterial TGase; lane 3, soybean peroxidase; lane 4, soybean peroxidase and bacterial TGase

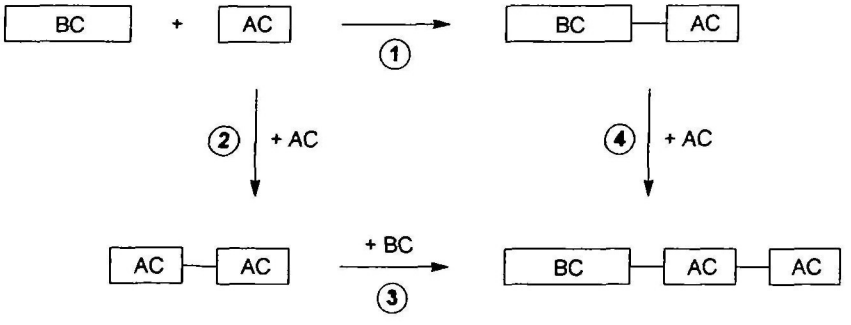
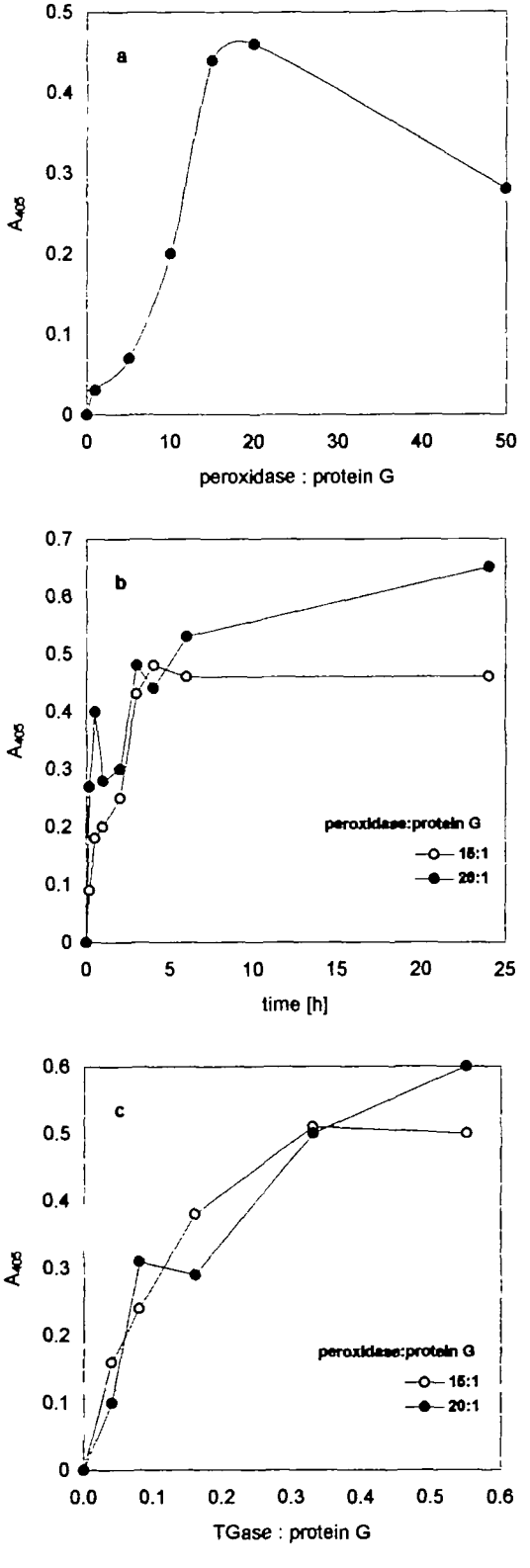


Fig. 2. **Conjugation of an antibody binding compound (BC) with an amplifying compound (AC) catalysed by transglutaminase.** Schematic representation of some possible products that may be expected if BC is provided with either reactive glutamine or lysine residues and AC with both amino acids.

TGase may preferably form conjugates consisting of protein G (strong lysine donor) surrounded by single peroxidase molecules (weak lysine donor). Nevertheless, aggregates consisting of protein G and peroxidase oligomers should be present in the reaction mixture as minor products.



Enzymic Coupling of Protein G and Peroxidase—Soybean peroxidase and protein G were incubated in 1 M Tris-HCl buffer, pH 7.0, at 37°C, at molar ratios in the range of 1:1 to 50:1. The amounts of conjugates formed were determined by immobilising the products onto IgG coated microtiter-plates and measuring the enzyme activity. The highest yields were obtained with 15- to 20-fold excess peroxidase present in the reaction mixture. The maximum values were obtained within 3–4 h (Fig. 4). Variation of TGase reveals an optimum at 30–50% of the protein G concentration. Fur-

Fig. 4. **Parameters influencing the enzymic formation of protein G peroxidase conjugates.** Mixtures containing protein G, peroxidase and TGase (ratio of protein G to TGase of 1:0.75) were incubated in 1 M Tris-HCl buffer, pH 7.0, at 37°C for up to 24 h unless otherwise stated. After immobilisation of the 1:90 diluted mixtures onto IgG coated microtiter plates, absorbance was measured at 405 nm as described in "MATERIALS AND METHODS." a, molar ratio of peroxidase to protein G; b, incubation time; c, TGase concentration expressed as the molar ratio of protein G to the coupling enzyme (incubation time 3 h).

TABLE III. **Effect of buffer system and pH on the enzymic coupling of protein G and peroxidase.** Absorbances measured at 405 nm are given as described in "MATERIALS AND METHODS."

200 mM buffer	pH	protein G:peroxidase	
		1:15	1:20
MOPS	7.5	0.25	n.d.
Sodium phosphate	6.0	0.55	0.70
	6.5	0.73	0.70
	7.0	0.74	0.95
Tricine	7.5	0.70	n.d.
Triethanolamine	7.5	0.17	n.d.
Tris	7.0	0.38	0.48
	7.5	0.31	0.49
	8.0	0.37	0.47
	8.5	0.26	0.25

n.d., not determined.

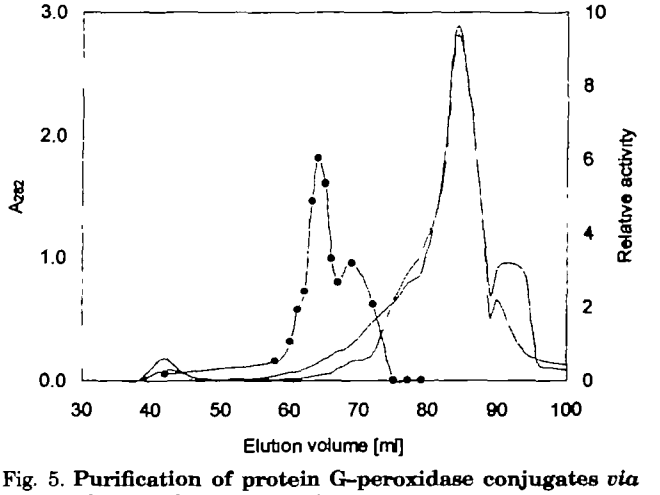


Fig. 5. **Purification of protein G–peroxidase conjugates *via* size exclusion chromatography.** Reaction mixtures containing protein G, soybean peroxidase and bacterial transglutaminase in a molar ratio of 1:15:0.3 in phosphate buffer, pH 7.0, were separated on a 60 cm × 1.6 cm Superdex 200 column. — U.V. absorption at 282 nm of a reaction mixture with TGase; –•–•– U.V. absorption at 282 nm of a reaction mixture without TGase; –●– conjugate activity expressed as ΔE/h.

ther enhancement of the coupling enzyme did not considerably increase the formation of conjugate. However, the buffer system and pH have a considerable influence on the cross-linking reaction. The most effective coupling took place in sodium phosphate at pH 7.0 or Tricine-HCl buffer at pH 7.5 (Table III).

Isolation of Conjugate—The protein G–enzyme conjugates were prepared in 0.2 M phosphate buffer, pH 7.0, under the optimized conditions outlined above. Size exclusion chromatography on Superdex G 200 enabled both the isolation of pure conjugate and the recovery of unreacted biomaterial in a single step. However, in contrast to the chromatographic behaviour of the single proteins, the unreacted peroxidase and protein G eluted together, almost unseparated, evoking a broad protein peak between 75 and 90 ml (Fig. 5).

The fractions containing the conjugate molecules were analysed again by binding to immobilised rabbit IgG and measuring their enzyme activity. Additionally, the total amounts of protein G and peroxidase in each fraction were compared with those of corresponding fractions from a control reaction without bacterial TGase separated in the same way as the reaction mixture. All measurements revealed that most of the conjugates were eluted between 59 and 71 ml.

The combined conjugate fractions were desalted, lyophilized, and examined by PAGE and isoelectric focusing, then blotted onto NC membranes and analysed for bifunctionality. Electrophoresis showed the conjugate samples to be free of starting materials and cross-linking enzyme (Fig. 6a, lane 6). A diffuse band at high molecular weight indicates that a mixture of different coupling products was generated by bacterial TGase. In the presence of thiol compounds such as mercaptoethanol, a protein with M_r of 66 kDa could be observed that obviously derived from a 1:1 coupling of peroxidase and protein G (not shown). The isoelectric points (pI) of peroxidase and protein G differ only slightly at 4.2 and 4.5, respectively (Fig. 6b). Accordingly, the pI values of the conjugate varied in the range of 4.3–4.6 (lane A6). Blotted on NC membranes, the proteins concerned showed peroxidase (lane B) and protein G activities (lane C).

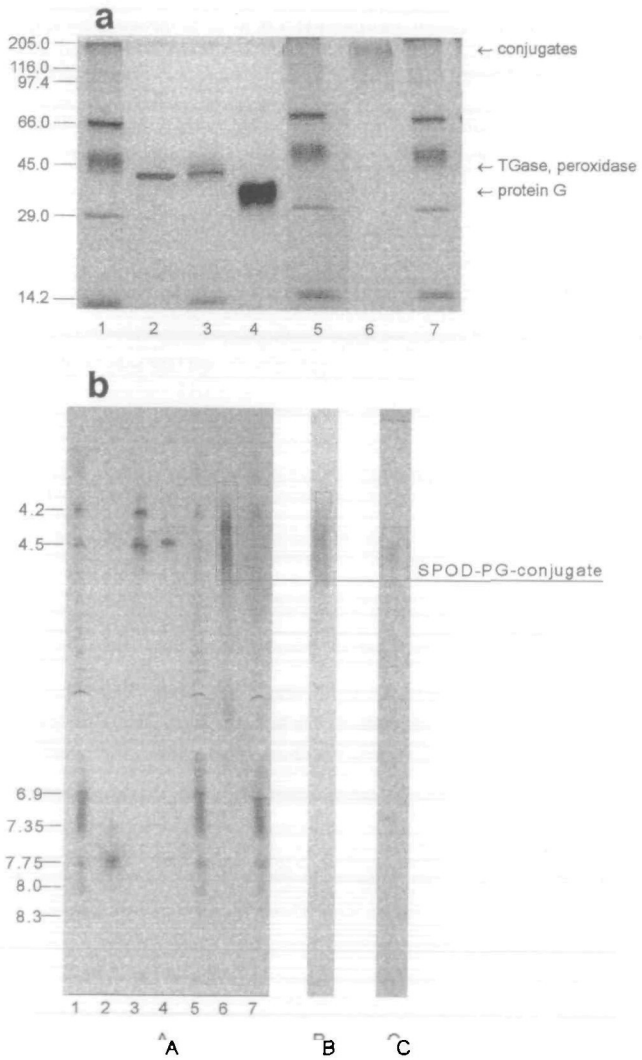


Fig. 6. **a: SDS–polyacrylamide gel electrophoresis of purified protein G–peroxidase conjugates.** Lanes 1, 5, 7, molecular weight marker proteins; lane 2, bacterial transglutaminase; lane 3, soybean peroxidase; lane 4, protein G; lane 6, protein G–peroxidase conjugates (combined fractions with elution volumes of 60–67 ml, see Fig. 5). **b: Isoelectric focusing (A) and western blots (B, C) of purified protein G–peroxidase conjugates.** A: Lanes 1, 5, 7, protein markers; lane 2, bacterial transglutaminase; lane 3, soybean peroxidase; lane 4, protein G; lane 6, protein G–peroxidase conjugates (combined fractions with elution volumes of 60–67 ml). B: Western blot of protein G–peroxidase conjugates (lane 6) using peroxidase staining as described in "MATERIALS AND METHODS." C: Western blot of protein G–peroxidase conjugates (lane 6) using protein G staining as described in "MATERIALS AND METHODS."

CONCLUSIONS

Enzyme immuno assays (EIA) are among the most sensitive methods for measurements of biological materials. The use of conjugates is necessary for all procedures, and their quality determines the sensitivity of an EIA. Up to now, the preparation of conjugates has been carried out with chemical linkers that often cause many problems. An enzymic procedure may overcome some of the inherent limitations of conjugate synthesis.

Transglutaminases are the most suitable enzymes for protein cross-linking. They catalyse the formation of stable isopeptide bonds between the glutamine side chains of a protein or peptide and a primary amine which is, in the case of a second protein or peptide, the ε-amino group of a lysine residue. In contrast to coupling procedures using chemical linker molecules, protein denaturation combined with activity loss should become negligible. This enables the recycling of unreacted materials.

Unfortunately, the access of transglutaminases to potential glutamine donor proteins is rather limited. Additionally, a comparison of the reactive sites of mammalian TGase substrates did not reveal a general consensus sequence (*2*), making substrate screening necessary. For the extensive investigations in this work, we used TGase from *Streptoverticillium* sp. since, in our experience, its specificity is comparatively low.

Numerous commercial protein preparations were analysed by the enzymic incorporation of a fluorescent or biotinylated amine (which permits the detection of glutamine donor proteins) and a fluorescent glutamine peptide (which

indicates lysine donor proteins). Immunoglobulin-binding protein G was identified as an excellent substrate of bacterial TGase. The enzymes examined are either very weak TGase substrates or do not react at all. Horseradish peroxidase, alkaline phosphatase from *E. coli* and β-galactosidase from *Aspergillus oryzae* could not be labeled, at least under the conditions used. Other enzymes, such as peroxidase from *Arthromyces ramosus*, are poor substrates. Nevertheless, soy bean peroxidase, *E. coli* β-galactosidase and alkaline phosphatase from bovine intestinal mucosa were identified as useful glutamine and lysine donor molecules. Including the known TGase substrate lysozyme (*17*), important enzymes have now been characterised which may be used in the enzymic preparation of conjugates.

With protein G and soy bean peroxidase, we prepared for the first time TGase coupled conjugates that could be clearly characterised by their bifunctionality. Separation of the reaction mixtures *via* size exclusion chromatography yielded conjugate preparations free of unreacted materials.

Analysis of the combined fractions revealed only a small portion of protein G conjugated with peroxidase, and, in accordance with this, the yield of conjugate calculated on the basis of active protein G was about 0.1%. Aggregates of protein G with up to 4 peroxidase molecules (average 3 molecules) had been formed. Obviously, protein G, which should be a stronger lysine donor than the enzymes, could not sufficiently suppress cross-linking reactions between the enzymes, or protein G was able to bind two or more single enzyme molecules leading to aggregates consisting of protein G enveloped, by peroxidase. The latter case also suggests the formation of conjugates having lost the ability to bind immunoglobulins by steric hindrance. This contingency would explain, at least in part, the low product yield.

Further investigations are necessary in which the reaction conditions are varied by enhancing the osmotic pressure (*i.e.* by increasing the salt concentration or adding of saccharides) and altering the coupling procedure, for example by immobilizing the TGase or one of the conjugate compounds.

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