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Review

Galactosylation as a tool for the stabilization and immobilization of proteins

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ABSTRACT

This paper presents a brief overview of the role that the carbohydrate moieties of biologically active glycoproteins play in the stabilization and oriented immobilization of these proteins on solid supports. The synthetic galactosylation of hydrophobic areas or their surroundings on the protein surface improves the structural stability of native proteins against inactivation by the interaction of water with hydrophobic clusters. The lowering of the degree solvation of tyrosine residues in galactosylated trypsin and the model substance N-carbobenzoxy-L-glutamyl-L-tyrosine was proved by Raman spectroscopy. D-Galactose residues can be selectively oxidized, either with periodate or enzymatically, and the aldehyde groups thus formed are used for the immobilization of glycoproteins on solid supports with hydrazide groups under mild conditions.

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1. INTRODUCTION

Biospecific interactions are fundamentally important for the maintenance of cells in their normal physiological state and for the selective control of pathological deviations from this state [1]. The combination of several complementary binding sites on the surface of biologically active compounds, *e.g.* enzymes or antibodies, makes the formation of a number of biospecific complexes possible. Biospecific adsorbents can be used for the efficient isolation and determination of many biolog-

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ically active molecules, for their oriented immobilization onto solid supports and they are also useful for the study of natural processes. The advantage of using immobilized enzymes for studying the stability and activity of biospecific adsorbents is the simple determination of enzymatic activity.

Protein inactivation under the influence of various denaturing actions in the surrounding microenvironment is usually considered to be a two-step process [2]:

$$N \rightleftarrows D \rightarrow I$$

where, N, D and I are the native, reversibly denaturated and irreversibly inactivated forms of a protein, respectively. A general strategy of protein stabilization should be based on the inhibition of the first step of the inactivation mechanism, *i.e.* the unfolding of the protein molecule [3]. Blanco *et al.* [4] have developed strategies for the stabilization of trypsin by multipoint covalent attachment on glyceryl–Sepharose 6B gels after various degrees of periodate oxidation. One of several advantages of the immobilization system described is that the geometric congruence between the enzyme and support surfaces is very intense.

The contact of non-polar clusters located on the surface of protein molecules with water destabilizes the protein. These non-polar amino acid residues play an important role *in vivo*, as they allow proteins to bind via hydrophobic interactions to other proteins, *e.g.* to form multi-enzyme complexes, to lipids in biological membranes and to cell walls. Mozhaev *et al.* [5] described a reduction of the non-polar surface area, which resulted in the stabilization of proteins. This stabilization is observed when comparing the *in vitro* structure and stability of mesophilic and thermophilic proteins. The stabilization of the protein structure by sugars and the immobilization of glycoproteins, *e.g.* enzymes and antibodies, through the carbohydrate moieties are briefly discussed in this review.

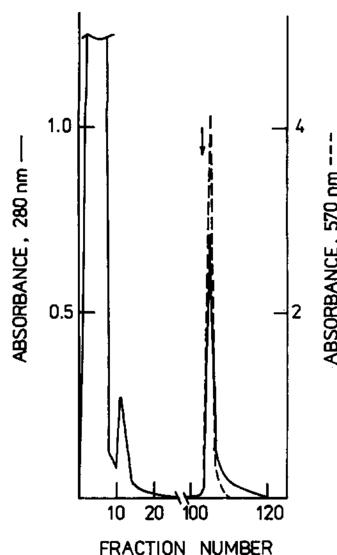


Fig. 1. Purification of CPY by bioaffinity chromatography on Separon–Gly-Gly-*p*-aminobenzylsuccinic acid (2.6 μmol of inhibitor per 1 g of dry adsorbent). The column (5 ml) was equilibrated with 0.01 *M* sodium acetate, pH 5.0. The column, after the application of the autolysate (50 ml), was washed with 1000 ml of 1 *M* sodium chloride–0.01 *M* acetate, pH 4.3. The elution of CPY was performed by 0.01 *M* phosphate buffer, pH 7.0. Peptidase activity was determined using N-carbobenzoxy-L-phenylalanyl-L-alanine as substrate (absorbance at 570 nm).

2. DEPENDENCE OF GLYCOPROTEIN STABILITY ON CARBOHYDRATE MOIETIES

For the application of biospecific adsorbents or biological catalysis, the economic feasibility of the process depends on the combination of efficiency and stability. The study of carboxypeptidase Y (CPY) showed the positive influence of carbohydrates on the stabilization and oriented immobilization of glycoproteins.

Fig. 1 shows the use of Gly-Gly-*p*-aminobenzylsuccinic acid–Spheron for the isolation of CPY from autolysates prepared from various kinds of yeast [6]. Enzymes with different molecular masses were isolated [7].

Table 1 was published previously by Margolis *et al.* [8]. In 1978 they showed the existence of multiple forms of CPY from *Saccharomyces cerevisiae*, with molecular masses varying from 49 000 to 65 500. In accordance with these workers, Turková *et al.* [7] also found that all carboxypeptidases which differ considerably in molecular mass have identical amino acid compositions in their acid hydrolysates. Table 2, also published by Margolis *et al.* [8], shows that the differences in isolated CPY are due to the differences in the content and composition of the carbohydrate moieties. Chu and Maley [9] showed a decrease in the stability of CPY with decreasing car-

TABLE 1

MOLECULAR MASSES OF VARIOUS CPY PREPARATIONS [8]

Preparation	Enzyme source[a]	Molecular mass[b]
1	Fleischmann	49 000
2	Fleischmann	52 000
3	Fleischmann	53 610[c]
4	Anheuser-Busch	64 000
5	Anheuser-Busch	64 000
6	Anheuser-Busch	65 500

[a] Commercially available compressed baker's yeast prepared by the indicated companies was used.
[b] Molecular mass determined by sodium dodecyl suphate–polyacrylamide gel electrophoresis.
[c] Determined by sedimentation equilibrium.

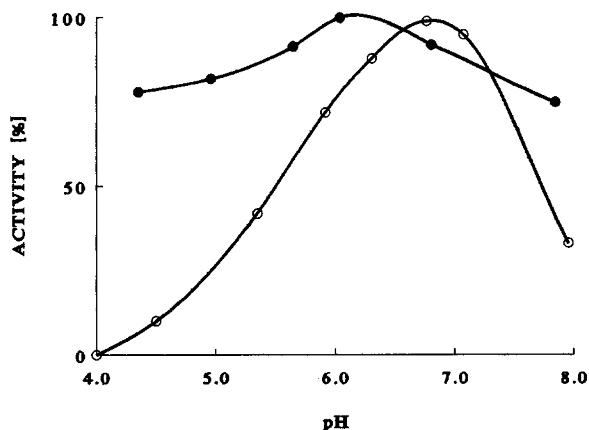


Fig. 2. pH rate profiles of peptidase activities of immobilized (●) and native (○) CPY. The activity was measured in 50 m*M* 2-(N-morpholino)ethanesulphonic acid buffer.

bohydrate content. The importance of carbohydrates in a molecule of acid phosphatase for its thermal stability and protection against proteolytic digestion was shown by Barbarič *et al.* [10]. Koyama *et al.* [11] established differences among rat alkaline phosphatases from various organs using serial lectin bioaffinity techniques. The purpose of their study was to establish the basis for studies on the importance of sugar chains in glycoproteins.

Fig. 2 shows the pH profile of peptidase activity of native CPY and of CPY attached to concanavalin A (Con A)–Spheron. The interaction of the carbohydrate moiety of CPY with the immobilized lectin Con A was used for the oriented immobilization described by Hsiao and Royer [12]. CPY, after adsorption onto Con A–Spheron, was cross-linked with glutaraldehyde [6]. Hsiao and Royer [12] immobilized CPY using amino, carboxyl and phenolic groups on the enzyme surface. A possible explanation for the low yields which they obtained could be that the amino acid chains in the glycoprotein were shielded by carbohydrate spines which projected from the surface of the enzyme molecule. When CPY was adsorbed onto immobilized Con A followed by cross-linking with glutaraldehyde, the bound enzyme retained 96% of the native catalytic acitivity and showed very good operational stability.

TABLE 2

CARBOHYDRATE ANALYSES OF SEVERAL PREPARATIONS OF CARBOXYPEPTIDASE Y [8]

No sialic acid, neuraminic acid, galactosamine, fucose or pentoses were detectable.

Preparation	Molecular mass	Total hexose (%)	Mannose[a] (%)	N-Acetyl-glucosamine (%)
3[b]	53 000	9	82	18
4[c]	64 000	15.4	76.1	23.9
5[c]	64 000	12.8	92.2	7.8
6[c]	65 500	20.1	94.1	5.9

[a] Expressed as percentage of total hexose.
[b] Fleischmann baker's yeast.
[c] Anheuser-Busch baker's yeast.

3. CARBOHYDRATE MOIETY AS A TOOL FOR ORIENTED IMMOBILIZATION OF BIOLOGICALLY ACTIVE COMPOUNDS

To prepare an efficient biospecific adsorbent for Con A, ovalbumin (OA) was immobilized using an anti-ovalbumin fraction of immunoglobulin G (IgG) that reacted with the antigen determinants located in the protein part of OA only [13]. Fig. 3 shows the isolation of OA antibodies from rabbit antiserum by bioaffinity chromatography on a column of OA immobilized via only one of its carbohydrate moieties after periodate oxidation to a hydrazide derivative of cellulose [14].

The immobilization of glycoproteins through

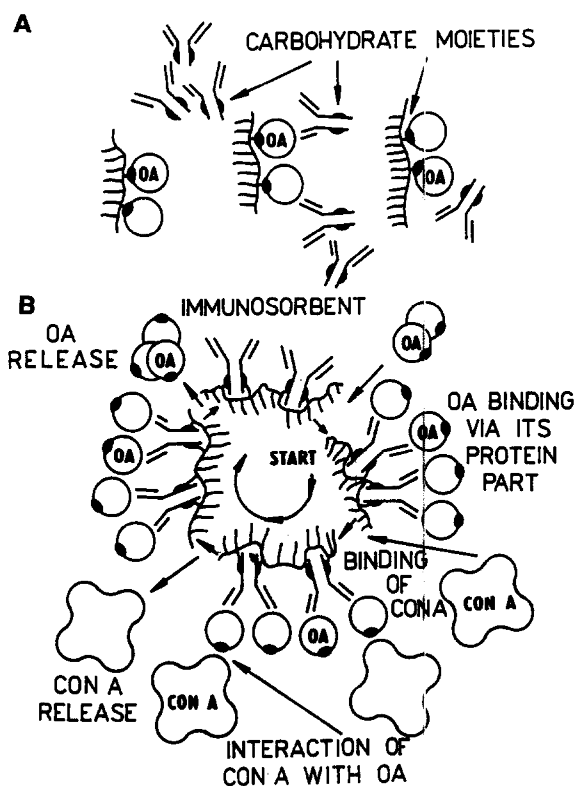


Fig. 3. Schematic diagram of (A) IgGs against OA using immunoaffinity chromatography on cellulose with OA attached via its carbohydrate moiety and (B) use of this oriented immobilized IgG (via the carbohydrate moiety on the hydrazide derivative of cellulose) after the adsorption of OA for the isolation of Con A.

their carbohydrate moieties is useful for enzymes and antibodies because their active complementary binding sites are located on their protein moieties. Therefore an immunoglobulin fraction containing antibodies against the protein moiety of OA was oxidized by periodate and bound via its carbohydrate moiety to cellulose hydrazide. OA was adsorbed onto this immunoadsorbent by its protein moiety, whereas its carbohydrate part remained free for interaction with Con A. The molar ratio of immobilized IgG to adsorbed OA molecules was 1:1.7, about 85% efficiency for the theoretical column capacity. Con A could be repeatedly bound and eluted. The specificity of the Con A–OA interaction was verified. It was shown that Con A did not adsorb onto the original support with immobilized immunoglobulins only. As free IgG reacts

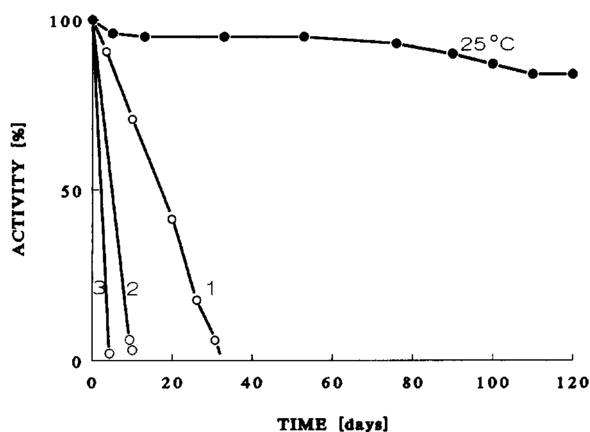


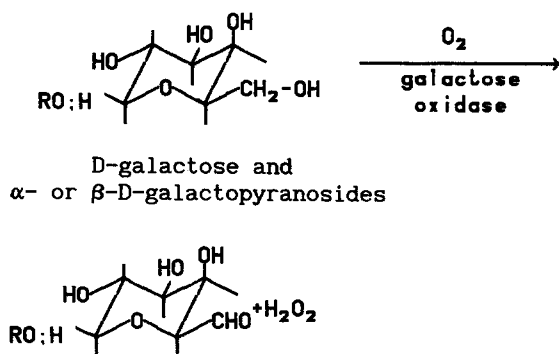
Fig. 4. Stability of immobilized (●) and soluble (○) GO. The enzyme was kept in phosphate buffer (pH 6.5) at 25°C. The concentrations of soluble GO were: (1) 1; (2) 0.1; and (3) 0.01 mg/ml.

with Con A in solution, this finding may be considered as evidence that IgG is immobilized via its Fc part and its carbohydrate moieties are not accessible for the interaction with Con A. Con A and OA were repeatedly bound and eluted and thus the chosen IgG–OA–Con A model strongly suggests the applicability of the principle of oriented immobilization for the preparation of various adsorbents suitable for the purification of different compounds in satisfactory yields and with good reproducibility.

4. GLUCOSE OXIDASE IMMOBILIZED THROUGH ITS CARBOHYDRATE MOIETY ACTIVATED BY CHEMICAL OR ENZYMIC OXIDATION

Zaborsky and Ogletree [15] have shown the great advantage of the immobilization of glucose oxidase (GO) from *Aspergillus niger* through its carbohydrate moiety after periodate oxidation to water insoluble *p*-aminostyrene. Fig. 4 shows the stability of GO from *Penicillium vitale* coupled after periodate oxidation to the hydrazide derivative of polyacrylate-coated glass [16] at 25°C [16,17]. The stability of the soluble enzymes was determined at concentrations of 0.01, 0.1 and 1 mg/ml.

Active aldehyde groups can be generated in glycoproteins or in solid supports containing D-galactose or D-galactopyranosides by the oxidation catalyzed by galactose oxidase. Toxic hydrogen peroxide can be removed with catalase:



Catalase catalyzes the following reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Fig. 5 shows the time course of the formation of the aldehyde group after the oxidation of GO from *Aspergillus niger* by galactose oxidase in the presence of catalase [18]. Oxidation of the enzyme did not change its activity. The oxidized enzyme was coupled to hydrazide derivatives of Sepharose and O-α-D-galactosyl–Spheron. Fig. 6 shows a comparison of the thermal stability of native GO and GO coupled to the hydrazide derivative of O-α-D-galactosyl–Spheron.

Galactose oxidase, after periodate oxidation, was coupled to hydrazide derivatives of cellulose and glass [19]. Immobilized enzymes have well known advantages and can be used repeatedly. The same conclusion has been reached by Solomon *et al.* [20], who used galactose oxidase and neuraminidase co-immobilized to a hydrazide support. They showed the possibility of the continuous oxidation of antibodies.

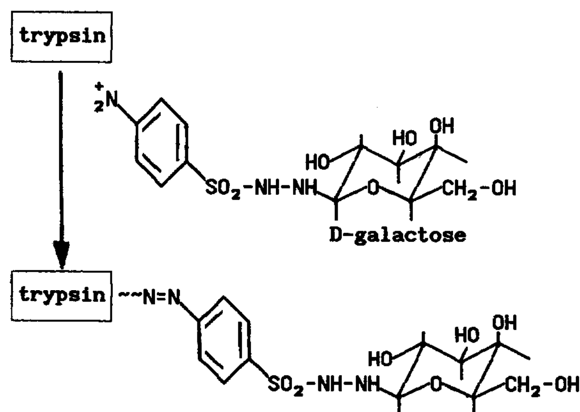
5. GALACTOSYLATION OF THE NON-POLAR SURFACE AREA OR SURFACE RESIDUES NEAR NON-POLAR CLUSTERS OF TRYPSIN

Non-polar amino acids on the surface of proteins are often organized into hydrophobic surface clusters. The contact of non-polar residues with water is thermodynamically disadvantageous and is harmful to the protein stability *in vitro*. Hence, a reduction of the non-polar surface area should stabilize the proteins. Hydrophilization of the non-polar surface area is, according to Mozhaev *et al.* [21], the most simple and reliable approach to artificial stabilization. As is shown in Fig. 7, hydrophilization can be performed by two ways. First, the hydrophobic amino acid of the protein surface can be modified directly by a hydrophilic reagent. An example of the successful application of this approach is the incorporation of amino groups by Mozhaev *et al.* [5] onto the surface tyrosine residues of trypsin, resulting in a hundred-fold stabilization of the enzyme. Secondly, any surface residues located near non-polar clusters on the protein surface may be modified by the hydrophilic reagent. The hydrophilic modifier can shield the clusters from contact with water. This approach has been shown for chymotrypsin, where amino groups were alkylated with glyoxylic acid [22].

With regard to the results obtained, D-galactosyl residues were coupled to trypsin using both methods to increase its hydrophilic character and to make its immobilization more suitable.

The galactosylation of trypsin was performed by azo-coupling tyrosine with diazotized 4-amino-N′-(D-galactopyranosyl) benzenesulphonohydrazide [23,24], as shown below.

The activity of trypsin containing covalently bound D-galactosyl residues determined with N-benzoyl-D,L-arginine-*p*-nitroanilide as a substrate was not changed compared with that of the unmodified enzyme. The thermal stability of galactosylated trypsin increased twelve-fold at 70°C.



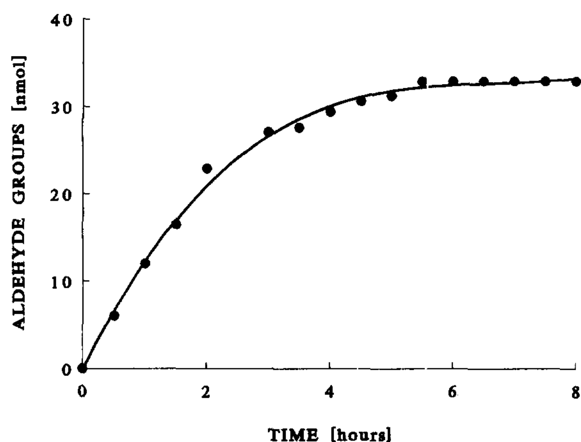


Fig. 5. Time course of GO oxidation by galactose oxidase in the presence of catalase. GO (4 mg) dissolved in 1.5 ml of 0.1 *M* phosphate buffer (pH 7.0) was oxidized with 250 μl of galactose oxidase (activity 8.8 U/ml). The H_2O_2 formed was removed with 100 μl of peroxidase (activity 50 U/ml). The reaction course was monitored from dye developed using 50 μl 0.001 *M* 3-methyl-2-benzothiazoline hydrazone, and 100 μl 0.025 *M* 3-(dimethylamino)benzoic acid.

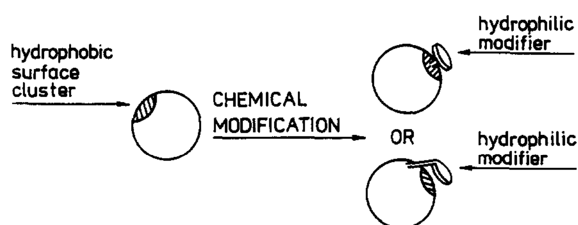


Fig. 7. Schematic diagram of two methods of protein hydrophilization by covalent modification.

The galactosylation of tyrosine residues was studied by Raman spectroscopy [23]. Raman scattering is a phenomenon of spectral shift in scattered light due to the vibrational motion of molecules. Fig. 8 shows a standard experimental set-up used for the measurement of Raman spectra. The cuvette with the sample is irradiated by monochromatic laser light. Scattered light is collected at right angles and its spectrum is recorded by the grating monochromator and the detection system, which consists of the photomultiplier and photocounter. The Raman spectra of a molecule contain a set of bands corresponding to different parts of the molecule. To determine the differences between Raman spectra corresponding to unmodified and galactosylated tyrosine residues, N-carbobenzoxy-L-glutamyl-L-tyrosine was used as a simple model. Four spectra were compared: the spectrum of galactosylated dipeptides after coupling, the spectrum of the mixture of carbohydrate and dipeptide, the spectrum of dipeptide and the spectrum of carbohydrate used. The comparison of the bands corresponding to tyrosine in the mixture tyrosine plus carbohydrate and glycosylated tyrosine showed the decrease in the contact of water with the galactosylated tyro-

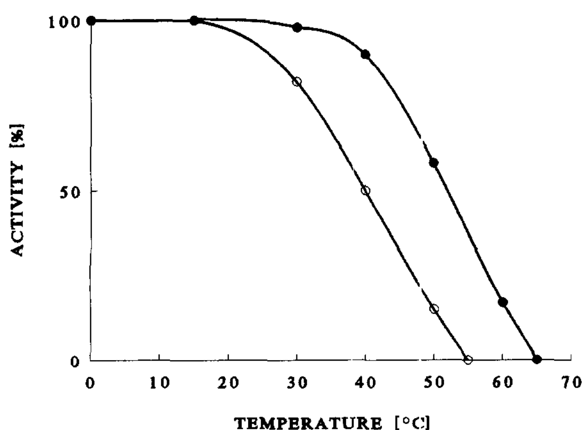


Fig. 6. Comparison of thermal stability of native GO (○) and GO coupled to the hydrazide derivative of O-α-D-galactosyl-Spheron (●). Enzyme samples were maintained at the indicated temperature in 0.1 *M* phosphate buffer (pH 7) for 50 min. The specific acitivity at 25°C determined by standard assay was taken as 100% for the calculation of relative activity. The concentration of native GO in the buffer was 56 and bound GO 45 mg/ml.

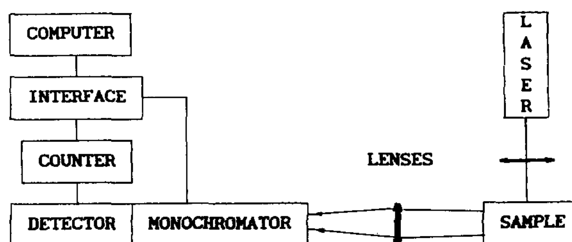


Fig. 8. Schematic diagram of Raman spectrometer.

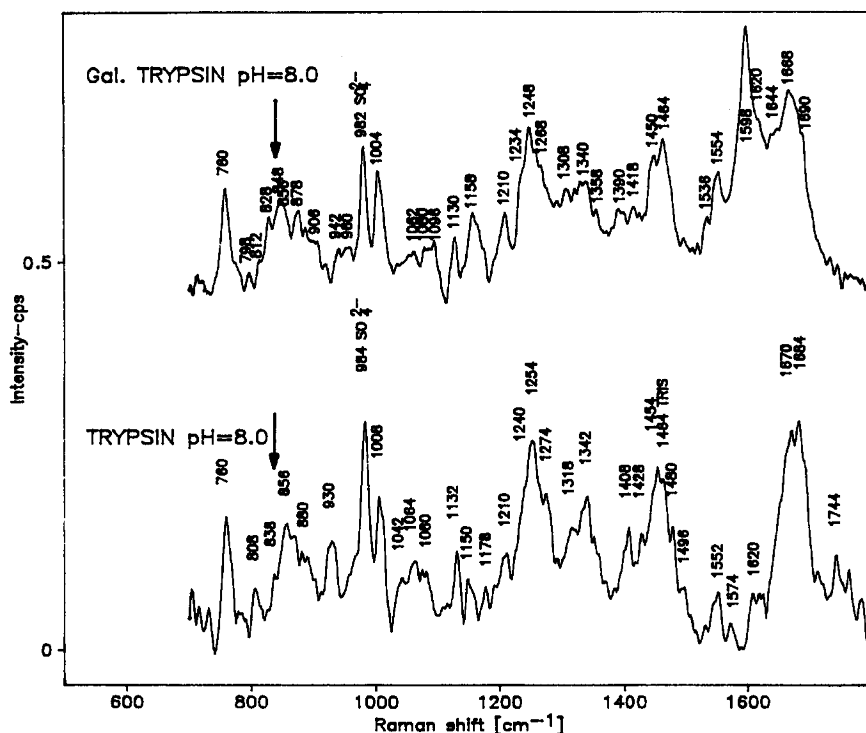
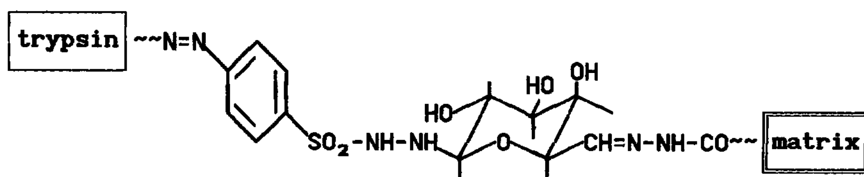


Fig. 9. Raman spectra of unmodified and galactosylated trypsins.

sine. Fig. 9 shows the Raman spectra of unmodified and galactosylated trypsin. The two close bands marked by arrows correspond to tyrosine residues which are sensitive to solvation. It is seen that the ratio of the first band to the second increases when the trypsin is galactosylated. This means that the galactosylation causes a lowering of the solvation of tyrosine residues in galactosylated trypsin. The modified trypsin was attached to the hydrazide derivatives of bead cellulose, porous glass and the membrane Immobilon through enzymatically oxidized galactosyl groups bound to the tyrosyl residues of the enzyme, as shown below.

The stability of galactosylated trypsin immobilized to the hydrazide derivative of cellulose increased 100 times at 70°C.

The second method of stabilizing trypsin is galactosylation by reductive amination; the surface amino groups of trypsin were modified in this way. The modification of the enzyme was carried out in the presence of a natural trypsin inhibitor, antilysin, at pH 8. Galactosylated trypsin and galactosylated trypsin inhibitor were isolated by gel chromatography on a Sephadex G-100 column without any change in their activity (Fig. 10). It is suggested that the linear form of D-galactose, which is in equilib-



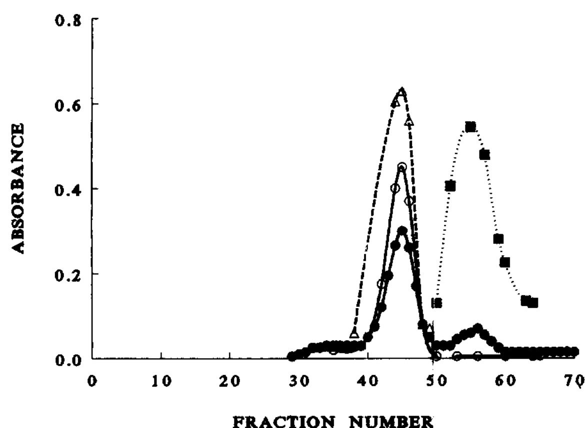


Fig. 10. Separation of galactosylated trypsin and galactosylated trypsin inhibitor on Sephadex G-100 equilibrated with 0.1 *M* glycine–HCl buffer (pH 2.5). After the application of sample, elution was performed with the same buffer. The flow-rate was 20 ml/h and the fraction volume 5 ml. Protein (●, A_{280nm}), protein activity of galactosylated trypsin determined by high-molecular-mass substrate–haemoglobin (○, A_{280nm}) and by low-molecular-mass substrate–N-benzoyl-D,L-arginine-*p*-nitroanilide (△, A_{410nm}); activity of galactosylated trypsin inhibitor (■, A_{410nm}).

rium with the cyclic forms participates in the reaction of the sugar with free amino groups of the enzyme. Fig. 11 shows the stability of soluble trypsin and galactosylated trypsin containing higher and lower amounts of coupled D-galactose.

Blanco *et al.* [4] studied the stability of trypsin

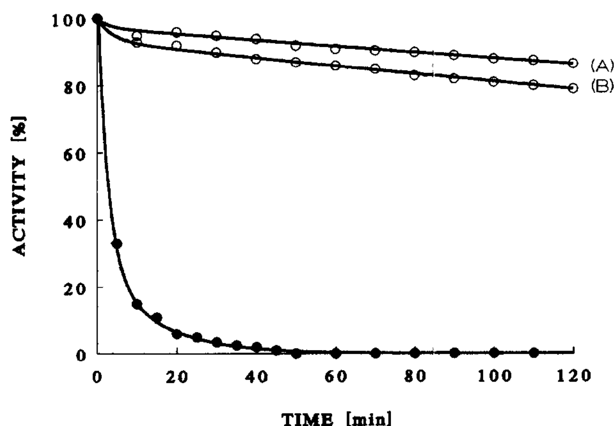


Fig. 11. Stability of soluble unmodified (●) and galactosylated (○) trypsins with higher (A) and lower (B) contents of coupled D-galactose. The enzyme was stored in phosphate buffer (pH 7.6) at 50°C.

attached by different numbers of lysine residues to aldehyde groups prepared by periodate oxidation of glyceryl–agarose. The stability of the immobilized enzyme increased with increasing number of coupled surface lysines. The attachment of seven lysine residues per trypsin molecule to activated agarose gave immobilized trypsin, which preserved 100% of the catalytic activity with a 5000-fold higher stability. A very important part in the preservation of catalytic activity of the derivatives was the presence of a competitive inhibitor, benzamidine, in the reaction medium during the immobilization. To protect a large area of the active site of trypsin after galactosylation, a natural trypsin inhibitor of molecular mass 6000 was used for biospecific complex formation during the galactosylation procedures.

To immobilize the galactosylated trypsin to hydrazide derivatives of glass and cellulose, the aldehyde groups formed by periodate and enzymatic oxidation by galactose oxidase were used. Highly active immobilized trypsins were prepared by these two methods.

6. CONCLUDING REMARKS

The glycoprotein CPY is an example of the favourable influence of the carbohydrate moiety on the stability and activity of biologically active proteins. As the carbohydrate content of CPY from various kinds of yeast increases, the protein stability also increases. High catalytic acitivity and good operational stability of immobilized CPY can be obtained by its adsorption onto the immobilized lectin Con A after cross-linking the biospecific complex with glutaraldehyde.

An efficient biospecific adsorbent for Con A, prepared by the adsorption of OA onto suitable IgG fractions coupled using oriented immobilization through their carbohydrate moieties to hydrazide-derivatized cellulose, is an example of bioaffinity chromatography. This technique gives satisfactory yields and good reproducibility. The immobilization of stable GO coupled to hydrazido-derivatized supports through the carbohydrate moieties shows the possibility of the activation of the carbohydrate moiety either chemically by periodate oxidation, or enzymatically using galactose oxidase.

Hydrophilization of the non-polar surface area of proteins can be used for stabilization by the galac-

tosylation of hydrophobic amino acids or of surface residues located near the non-polar clusters. The lowering of the contacts of galactosylated tyrosine residues in trypsin with water has been shown by Raman spectroscopy. Hence, galactosylation of suitable parts of protein surfaces stabilizes the native protein structure. Moreover, coupled galactose residues have been used for immobilization using chemical periodate oxidation or enzymatic oxidation with galactose oxidase. Immobilized galactose oxidase can be repeatedly used for the activation of galactosylated proteins. However, no method of stabilization or immobilization can be considered as universal.

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