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ENZYME COUPLING METHOD ON CALIBRATED NYLON SPHERES: APPLICATION TO THE SELECTIVE TRYPSINIZATION OF HISTONES IN CHROMATIN

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A new method consisting of a two-step activation was developed in order to covalently immobilize enzymes on calibrate nylon 66 spheres. This efficient method associates for the first time peptide bond cleavage and O-alkylation of the support. Optimal conditions for activation and protein coupling were defined, and immobilized trypsin was used to investigate the histone accessibility on chromatin. This approach, which allows us to degrade first progressively H1, indicates that H4 seems inaccessible both in relaxed and condensed chromatin.

Enzymes immobilized on water-insoluble carriers attract much interest because of their numerous advantages, such as an enhanced stability, an easy control of reactions, and the absence of residual enzyme and contamination in the digestion medium, which can disturb or prevent further studies (1,2). For these reasons, proteases immobilized on various supports were recently used to investigate the protein topography in nucleoproteic complexes (3-9). In our laboratory, the covalent coupling of enzymes on collagen membranes after an acyl-azide procedure (10) was successfully used to correlate the proteolysis of the most accessible proteic fragments in chromatin (3,8) and ribosome (6) with structural modifications. Recently, we have reported new methods to generate potentially reactive centres necessary for the coupling of proteases or antibodies on polystyrene or nylon calibrate spheres (7,11,12). Here we describe a considerable improvement of these procedures consisting of a two-step activation of nylon spheres by involving both peptide bond cleavage and O-alkylation of the support. These two approaches, which were till now adopted alternatively, lead to the formation of different chemical groupments, that are capable of interacting covalently with enzyme molecules. The choice of the conditions of nylon activation and enzyme coupling allows to control perfectly and modulate the activity of immobilized trypsin. Chromatin was selectively trypsinized and the role of histone tails in the stabilization of the solenoid was analysed. These regions are the major sites of post-translational modifications and potential sites of interactions either between nucleosomes or with non-histone proteins, which led to suggest a decisive role of these histone tails in chromatin condensation (2-4). Compared to previous studies using immobilized proteases in the study of chromatin structure,

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Abbreviations: TNBS, 2,4,6 trinitrobenzene sulfonic acid; TAME, p-toluene-sulfonyl -L-arginine methyl ester.

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enzymatic nylon spheres show a better ability of reproducibility, storage and reusing than collagen membranes (10) and avoid filtration and inhibitor addition necessary in using commercial trypsin-glass (9). This paper describes the preparation of tryptic spheres and the time-course of digestions of extended and condensed chromatin fibres.

MATERIAL AND METHODS

Activation of nylon 66 spheres. Nylon 66 spheres (3.2 mm-diameter, Precision Plastic Ball Co.) were immersed in a solution containing 0.5 N sulfuric acid in methanol, as previously described (7,11). After refluxing for 4 h 30, they were washed in water and then in 1% $NaHCO_3$ for 1 h 30. The spheres were then allowed to react for 1 h under constant stirring with glutaraldehyde, whose concentrations ranged from 0.25 to 16%. After being thoroughly washed to eliminate unreacted reagent, the spheres were ready for enzyme coupling.

Measurements of reactive groupments. The methoxycarbonimidoyl groups were measured as described (12,13), either immediately after the treatment by sulfuric acid in methanol, or after the covalent fixation of a small molecule, lysine. Two methods were used to measure amino groups after the first activation process. The first one is based on the reaction of the matrix with TNBS (14), and the second on the formation of an immobilized Shiff base between nylon and 2-hydroxy-1-naphtaldehyde (15). The values obtained are directly related to the coupling capacity.

Enzyme immobilization. Activated spheres were immerged in a solution of trypsin (Boehringer) at 1 mg/ml, using 0.1 M sodium borate, pH 8.5, as coupling buffer. The immobilization was performed at 25°C under constant stirring, for times varying from 2 min to 4 h. Then, the spheres were poured into a solution of 1% histidine in the coupling buffer for 4 h at 25°C, in order to inactivate the support. The spheres were washed in distilled water and in 1 M KCl for 1 h, to eliminate possible adsorbed enzymes. They were stored at 4°C either in 0.1% sodium azide or in 50 mM NaCl.

Activity measurements. The immobilized trypsin activity was determined by the rate of hydrolysis of TAME (Sigma), measured by the increase in absorbancy at 247 nm. 5 ml of the reaction mixture composed of 40 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.1, and 1 mM TAME, were maintained at 25°C under stirring. The reaction was initiated by adding the spheres to the mixture for 5 to 20 min. Spectra were continuously recorded using a Kontron Uvicon 930 spectrophotometer. The specific activities were expressed in nmoles of TAME hydrolysed per min and per sphere (with $\varepsilon = 540\ M^{-1} \times cm^{-1}$).

Isolation of chromatin and proteolytic digestion. Rat liver nuclei were isolated (16) and after solubilization for 1 min 30 by micrococcal nuclease (Sigma; 1.25 U/10^9 nuclei), chromatin was fractionated on 5-28.2% isokinetic gradients (17). After centrifugation, fractions containing 35 $\pm$ 5 nucleosomes per chain were pooled, extensively dialysed against TE buffer (10 mM Tris-HCl, 0.2 mM EDTA, pH 7.4) and then diluted at 2.4 A_{260}. Tryptic digestions were initiated by dipping 20 enzymatic spheres into 5 ml of the chromatin solution maintained at 25°C under constant agitation. The digestion of condensed fibres was performed in the presence of 50 mM NaCl in TE buffer. Aliquots (25 µl) were withdrawn at increasing incubation times and made 24 mM Tris-HCl, pH 6.8, 4% glycerol, 2% 2-mercaptoethanol and 0.08% SDS. They were denatured for 5 min, prior to being loaded on 16% polyacrylamide minislab gels (Idea Scientific), according to Laemmli (18). After running for 3 h, the gels were stained with 0.05% Coomassie blue.

RESULTS AND DISCUSSION

Protein covalent coupling.

Native nylon free end groups must be pretreated to generate potentially reactive centres necessary for enzyme coupling. The new activation method we have developed consists of a treatment by sulfuric acid in methanol, followed by the action of glutaraldehyde, as described in Materials and Methods. In this two-step method, the

first activation process involves two of the approaches generally adopted for the immobilization of enzymes on nylon (19)(Figure 1).

On one hand, in the presence of sulfuric acid in methanol, the secondary amide bonds of the nylon were alkylated to yield the corresponding imidate salts. The treatment then results in O-alkylation of some peptide bonds and the enzyme can react directly through mobil hydrogens with the imidate salts of the nylon (Figure 1A). The measurement of the methoxycarbonimidoyl groups after the covalent binding of lysine indicate that only about 3% of the groups formed by the acidic treatment in methanol (340 µmoles of groups/g of sphere) were allowed to react with amino groups. That small yields of immobilized molecules were obtained is not surprising since it has been noted that direct coupling in this manner does not result in the most active bound enzymes (20). This is due either to a problem of accessibility of the goups to the ligand or more likely to their short life duration.

In the second mechanism involved, the nylon is partially depolymerized by cleaving some of its secondary amide linkages, and liberates aliphatic amino and carboxyl groups. The estimation of the resulting free amino groups, made by two different methods (14,15) giving identical values, indicates that 4 µmoles of groups/g of sphere were formed. The presence of methanol improves by about 25% the quantity of reactive groups liberated, compared to a treatment with aqueous sulfuric acid (3 µmoles/g). Amino groups can react with an excess of the bifunctional agent glutaraldehyde, and the enzymes are then bound to nylon by reaction with its free aldehyde groups (Figure 1B).

The respective contributions of each mechanism to the protein binding were difficult to determine because of the participation of the amino groups of the enzymes in the measurements. Hence, we studied the influence of various activation factors by determining the enzymatic activity of spheres after the enzyme covalent binding.

Enzymatic activity of nylon spheres.

Figure 2A shows the influence of the time of coupling on the tryptic activity, after a treatment with 2% glutaraldehyde. For time varying from 15 min to 1 h, the

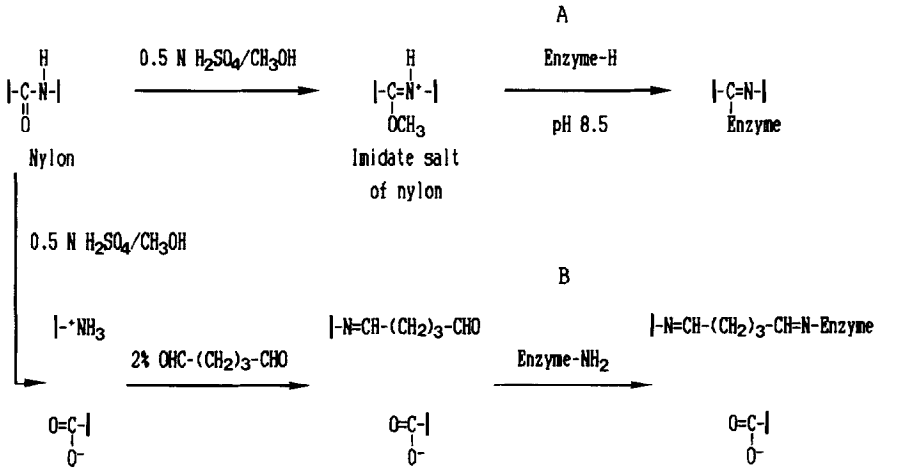

Figure 1 : Possible covalent binding of protein to nylon spheres. (A) O-alkylation of the nylon and reaction with mobil hydrogens of enzymes. (B) peptide bond cleavage and coupling through the liberated primary amino groups. The intermediates of the reactions (methoxycarbonimidoyl and amino groups) were measured as described in Material and Methods.

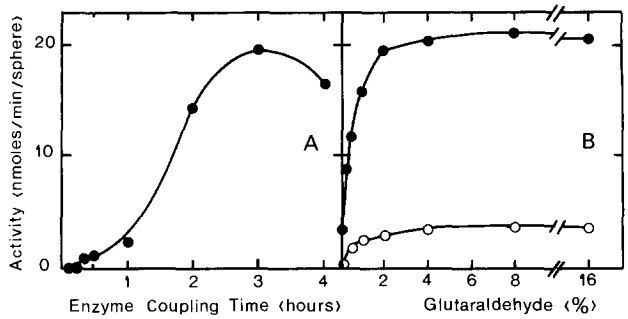

Figure 2 : Enzymatic activities of trypsin immobilized on nylon spheres. Enzyme coupling was performed in 0.1 M sodium borate, pH 8.5, on 3.2 cm-diameter spheres. (A) Influence of the duration of the enzyme coupling on the activity. Nylon sphere were first activated by sulfuric acid in methanol, and then by 2% glutaraldehyde. (B) Influence of glutaraldehyde concentration in the activation procedure on the activity. The first activation step was performed for 3 h with sulfuric acid in the presence of methanol (●) or not (○). Activity was expressed in nmoles of TAME hydrolyzed per minute per sphere.

activities are very low, suggesting that the coupling process is slow, and then their values increase proportionately with the coupling duration. The curve reaches a maximum when the coupling was performed for 3 h, and for longer durations, the activity slightly decreases. An optimal coupling time of 3 h was defined, which is not surprising since a coupling time of 3-4 h is generally necessary to allow the methoxycarbonimidoyl groups to react with amino compounds to produce stable amidine linkings (20).

The influence of glutaraldehyde concentration on the enzymatic activity is shown in Figure 2B. Whether the spheres were first treated by sulfuric acid in methanol or in water, a maximum was reached when 2% glutaraldehyde was used. That activity values are not significantly modified when the glutaraldehyde concentration increases could be explained by the fact that glutaraldehyde seems not to react as a dialdehyde but as a polymer resulting in an aldolic condensation of molecules (21)(Figure 3).

From the activity values obtained in absence of glutaraldehyde (3.5 nmoles of TAME hydrolysed/min/sphere), the participation of the methoxycarbonimidoyl groups to the total process is very low, taking into account the number of the groups formed during the activation. One explanation is that the reactive imidate forms could be transformed into non-reactive forms by hydrolysis during the coupling process.

But the enzymatic activities observed can not be explained by the simple addition of the two mechanisms described on Figures 1 and 3. It appears that concomitantly to its reaction with the amino groups resulting from the partial acid hydrolysis, glutaraldehyde might react with imidate salts (methoxycarbonimidoyl groups) to introduce new reactive centres onto the nylon. At this stage of our experimental studies, no mechanism can be proposed. Moreover, it must be recalled that alternative mechanisms for the reaction of glutaraldehyde on amino groups have also been suggested (19).

No significant differences in the enzymatic activities were observed after successive and repeated incubations, and from one lot of spheres to another. Besides the activities checked during an interval of three months only show a drop of the order of 6% and this stability makes the enzymatic spheres repeatedly reusable. Finally, this new coupling process which improves considerably the rate of fixation,

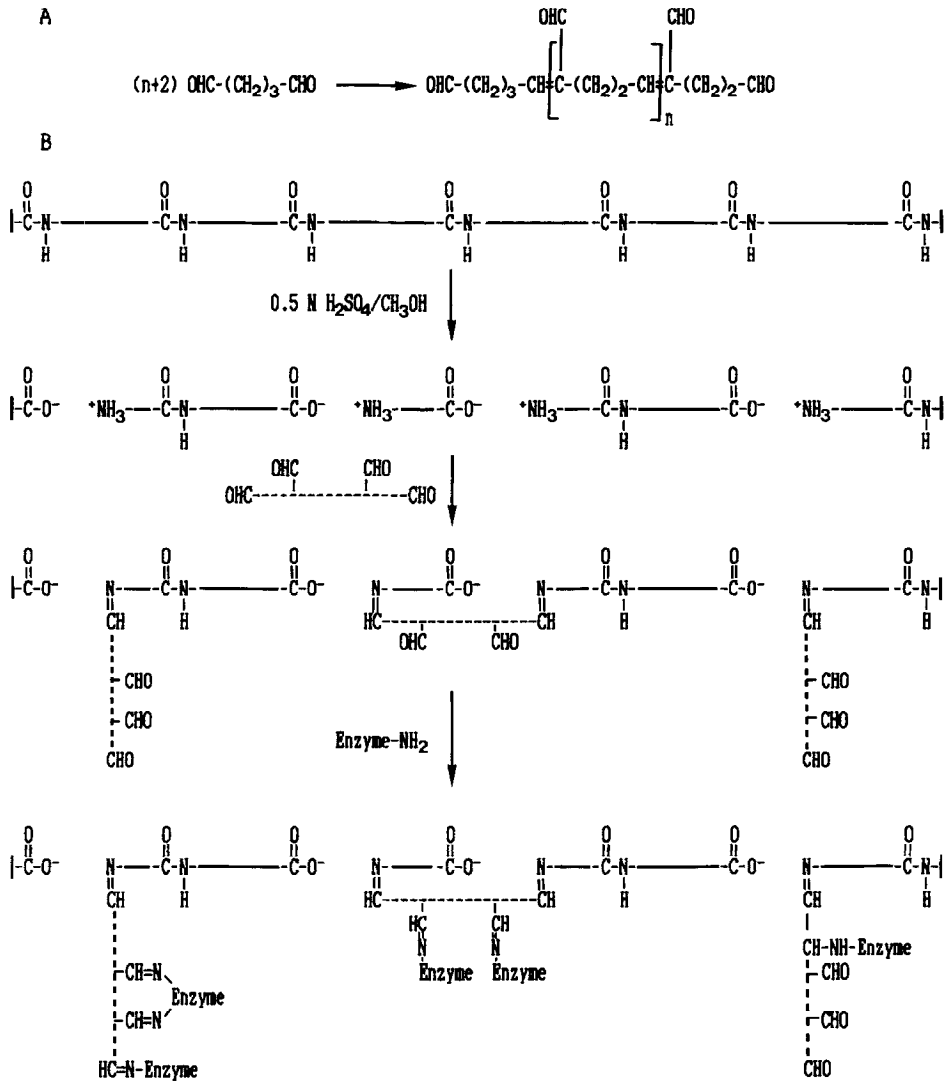

Figure 3 : Aldolic condensation of glutaraldehyde (A) and possible enzyme coupling to the glutaraldehyde derivatized nylon (B).

compared to the results obtained with other methods (7,11,12), allows to use spheres having higher specific activity, whereas small volumes of reaction medium are required (less than 50 µl/sphere). Besides, the activation processes are easy to perform, quick and unexpensive, and activated spheres could be prepared in advance in large amount. Recently, similar experiments were also carried out in order to immobilize different types of proteases and preliminary experiments gave encouraging results.

Proteolytic digestion of chromatin.

Trypsin was often used to study chromatin structure (2), since this enzyme specifically cleaves lysine and arginine residues, whose histone basic tails are mainly composed of, and the effects of the proteolysis were studied by various biophysical methods. Here we carried out proteolyses using trypsin immobilized

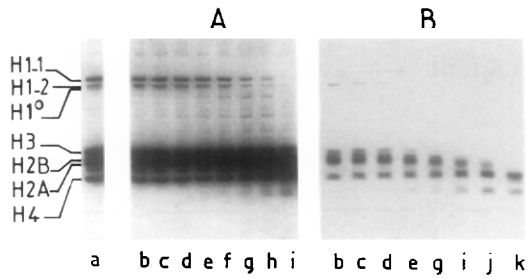

Figure 4 : Electropnoresis pattern of chromatin digested by trypsin immobilized on nylon spheres in 10 mM Tris-HCl, 0.2 mM EDTA, pH 7.4, containing 50 mM NaCl (B) or not (A). SDS-16% polyacrylamide gels were performed according to Laemmli (18). Native chromatin (a) and samples digested for: 10 min (b), 20 min (c), 30 min (d), 1 h (e), 1 h 30 (f), 2 h (g), 2 h 30 (h), 3 h (i), 4 h (j) and 5 h (k).

according to the method described, depending on the condensation of the chromatin fibres. At low ionic strength, chromatin exhibits a relaxed structure, in which histone basic tails are very accessible to proteases, but when 50 mM NaCl is added, it undergoes a condensed structure, the 30 nm fibre (see ref. 22 for recent reviews). Figure 4 shows the electrophoresis patterns obtained by digesting extended (A) or condensed (B) chromatin fibres. In both cases, histones H1 and H3 are rapidly attacked, but in the presence of 50 mM NaCl the histone proteolysis is much quicker. Thus, a 3 h digestion pattern in the absence of salts (Fig. 4 A,i) approximately corresponds to a 1 h digestion with 50 mM NaCl (Fig. 4B,e), suggesting that the basic tails are very exposed at the surface of the condensed fibres. Interestingly, in both cases, histone H4 appears to be very resistant to proteolytic cleavage, indicating that the sites are equally inaccessible in relaxed and condensed chromatin. This internal location could explain why the hyperacetylation of H4 has no or little effect on higher order structure while it induces important changes at the level of core particle and nucleosomes (23). No difference in the digestion patterns was observed when the samples were incubated 1 h at 25°C after the removal of the spheres prior to being loaded on the gels, which shows that no enzyme release occurs. Hence, all difference in protein composition among the digestion media was eliminated.

In conclusion, our results demonstrate that the use of immobilized enzymes constitutes a good approach to degrade progressively H1. This possibility might allow us to discriminate the contribution of H1 and H3 in stabilizing chromatin structure, as well as to understand the role played by the basic terminal tails of core histones. These aspects are currently being studied in our laboratories by correlating selective protein degradation with chromatin conformational changes monitored by sensitive immunochemical and electro-optical techniques.

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