Lait (1994) 74, 333-341
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Original article

Proteolysis of bovine β-casein by a plasmin-Sepharose conjugate

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(Received 1 April 1994; accepted 11 July 1994)

Summary — A plasmin-Sepharose conjugate (5.7 mg of active plasmin/g gel) was prepared by covalent immobilization of bovine plasmin (EC 3.4.21.7) on activated CH-Sepharose 4B, with formation of amide bonds between primary amino groups of plasmin and carboxyl-activated functions anchored to the gel. A strong decrease (60%) of the proteolytic activity of plasmin for a chromogenic substrate was observed during dialysis performed before immobilization. However, a high binding yield and a good preservation of the proteolytic activity of immobilized enzyme (95%) were then observed. The reactivity of free and immobilized plasmin with bovine β-casein (β-CN) were compared. Kinetic study of β-CN proteolysis indicated a perceptible loss of enzyme activity in plasmin-Sepharose conjugate compared to free plasmin (V_{max} = 3.8 10^{-2} and 53.5 10^{-2} OD unit/min, K_m = 1.2 10^{-4} et 6.2 10^{-4} mol/l, respectively). Electrophoretic study of the reaction mixtures showed that the proteolysis of β-CN f(1–105/7) by immobilized or free plasmin, producing β-CN f(29–105/7), succeeds the reaction of the whole β-CN. Microparticle-enhanced nephelometric immunoassay of residual β-CN, performed in reaction supernatants, indicated there is no significant influence of β-CN fragments (β-CN f(1–105/7) and mixture of C-terminal peptides β-CN (29-209), (106–209), (108–209)) on the proteolysis of the whole β-CN. As already observed for other proteases, an alteration of the enzyme charge during the immobilization process might be the cause of the slight modification of the catalytic activity observed in plasmin-conjugate.

immobilized enzyme / proteolysis / plasmin / β-casein

Résumé — Protéolyse de la caséine β bovine par un conjugué plasmine-sepharose. *Un conjugué plasmine-sepharose (5,7 mg de plasmine active/g de gel) est obtenu par immobilisation de la plasmine bovine (EC 3.4.21.7) sur du CH-sepharose 4B. Le couplage s'effectue par formation de liaisons amides entre les fonctions amines primaires de l'enzyme et les groupes carboxyles activés du gel. La dialyse de la plasmine avant couplage provoque une perte importante (60%) de l'activité protéolytique pour un substrat synthétique. Cette activité est en revanche bien conservée (95%) lors de l'immo-*

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bilisation de l'enzyme. La réactivité du conjugué avec la caséine β (β-CN) bovine est comparée à celle de la plasmine libre. L'étude cinétique de la protéolyse de la β-CN indique une diminution de l'activité de l'enzyme immobilisée par rapport à celle de la plasmine libre (V_{max} = 3,8 10^{-2} et 53,5 10^{-2} unité DO/min, K_m = 1,2 10^{-4} et 6,2 10^{-4} mol/l, respectivement). L'étude électrophorétique des milieux de réaction montre que, comme avec l'enzyme libre, la protéolyse de la β-CN entière par l'enzyme immobilisée est suivie de celle du fragment β-CN (1-105/7) qui libère le fragment β-CN (29–105/7). Le dosage de la β-CN résiduelle dans les surnageants de réaction est réalisé par immunonéphélémétrie microparticulaire. Il indique qu'il n'existe aucun effet inhibiteur mesurable des fragments de la β-CN (β-CN f(1–105/7) et mélange des fragments C-terminaux β-CN (29–209), (106–209), (108–209)) sur la protéolyse de la molécule entière par les plasmines libre et immobilisée. Comme pour d'autres protéases, une altération de la charge de l'enzyme, consécutive à son immobilisation, pourrait être responsable de la diminution de l'activité catalytique observée dans le conjugué plasmine-sepharose.

enzyme immobilisée / protéolyse / plasmine / caséine β

INTRODUCTION

Quality and some properties of milk products, such as cheese ripening and flavour, are directly dependent on the proteolytic activity of enzymes present in raw milk. Alkaline proteinase or plasmin (Humbert and Alais, 1979; Grufferty and Fox, 1988), originating from bovine blood (Kaminogawa *et al*, 1972; Halpaap *et al*, 1977; Reimerdes *et al*, 1981; Humbert *et al*, 1990) is one of these milk enzymes. Many properties of plasmin have been clearly established. It is a trypsin-like enzyme which cleaves polypeptidic chains after arginyl and lysyl residues (Weinstein and Doolittle, 1972). Plasmin hydrolyses β-casein (β-CN) and produces three main C-terminal fragments (cleavage at residues 28/29, 105/106 and 107/108) and the complementary N-terminal peptides (Eigel, 1977; Andrews and Alichanidis, 1983). These fragments of β-CN and whole β-CN coexist in bovine milk and form an intricate system (Eigel *et al*, 1979; Reimerdes and Herlitz, 1979).

This report describes the preparation of a plasmin-Sepharose conjugate, by covalent binding of bovine plasmin on activated CH-Sepharose 4B. Solutions of bovine plasmin are frequently unstable (Humbert, 1986). Immobilization of plasmin on a solid-phase such as Sepharose could allow its stabilization and the easy recovery of enzyme, after β-CN proteolysis, with its possible regeneration. The use of an immobilized enzyme for the proteolysis of β-CN by plasmin could also permit to obtain pure β-CN fragments, free from pollution by enzyme and inhibitor and more easily usable for the preparation of polyclonal and monoclonal antibodies.

MATERIALS AND METHODS

Biochemical reagents

Whole casein was extracted from a pool of fresh raw milk (Holstein breed) by three cycles of precipitation at pH 4.6, washing with distilled water and solubilization at pH 7, according to Nitschmann and Lehmann (1947).

β-Casein was obtained by fast protein liquid chromatography (FPLC) of whole casein on TSK-DEAE 5PW (Pharmacia, Uppsala, Sweden) in a 0.02 mol/l Tris-HCl buffer (pH 8.0) containing 4.5 mol/l urea and 0.01% of 2-mercaptoethanol with increasing ionic strength (from 0 to 0.35 mol/l NaCl).

Mixture of C-terminal peptides of β-CN (β-CN f(29–209), f(106–209) and f(108–209)) was prepared from plasmin-treated β-CN, by precipitation at pH 4.6, following the method of Wilson *et al* (1989).

Skimmed milk heated up to 95°C for 30 min was used to prepare a mixture of N-terminal peptides of β-CN (β-CN f(1–28), f(1–105/7) and f(29–105/7)) according to Aschaffenburg (1946). β-CN f(1–105/7) was purified by hydrophobic interaction FPLC on TSK-Phenyl 5PW (Pharmacia), following the methods of Paquet *et al*, (1988) and Girardet *et al* (1991).

Purified proteins were dialyzed against distilled water and freeze-dried. Polyacrylamide gel electrophoresis in 4.5 mol/l urea, 0.38 mol/l Tris-HCl buffer (pH 8.9) (Ng-Kwai-Hang and Kroeker, 1984), and sodium dodecyl sulphate-polyacrylamide gel (0.1% SDS, 0.38 mol/l Tris-HCl buffer (pH 8.9)) electrophoresis (Laemmli and Favre, 1973) were performed for all protein preparations. Amino acid composition of purified β-CN and β-CN f(1–105/7) was determined (Moore and Stein, 1963), after hydrolysis by 6 N HCl at 110°C for 24 h. Amino acid analysis was performed using Biotronik (Maintal, Germany) LC 3000 auto analyser.

Anti-β-CN antisera were obtained (El Bari *et al*, 1991) by immunizing rabbits with intradermal injections of purified β-CN in Freund's complete adjuvant (Behring, Marburg, Germany) for the first injection and incomplete adjuvant for following booster injections. The reactivity and specificity of antisera were assessed as previously reported.

Preparation of plasmin-Sepharose conjugate

Activated CH-Sepharose 4B (Pharmacia) was extensively washed in 1 mmol/l HCl (200 ml/g of gel) then suspended in 0.1 mol/l carbonate buffer (pH 8) containing 0.5 mol/l NaCl (5 ml/g of gel). Bovine plasmin (EC 3.4.21.7, 10 units/mg according to the first British standard 78/646, Sigma Chemical Co, St Louis, MO), previously dialyzed against the binding buffer, was added (15 mg/g of gel) and the mixture was stirred for 1 h at room temperature, followed by 4 h at 4°C. After the elimination of uncoupled plasmin by washing in the binding buffer, the remaining active ester groups of gel were blocked in 0.1 mol/l Tris-HCl buffer (pH 8) (5 ml/g of gel) for 1 h at room temperature. Plasmin-Sepharose conjugate was washed five times in 0.1 mol/l acetate buffer (pH 4) containing 0.5 mol/l NaCl, and 0.1 mol/l Tris buffer (pH 8) containing 0.5 mol/l NaCl, alternatively, then finally stored at 4°C in the binding carbonate buffer (5 ml/g of gel) containing 0.2% of NaN_3.

Comparative characterization of the plasmin-Sepharose conjugate

The binding yield of active plasmin in plasmin-Sepharose conjugate was determined by measuring (optical density (OD) at 405 nm) the proteolytic activity of bound plasmin for a chromogenic substrate (D-valyl-L-leucyl-L-lysyl-4-nitroanilide, Serva Feinbiochemica, Heidelberg, Germany) according to Rollema *et al* (1983).

Comparative kinetic study of the activity of plasmin-Sepharose conjugate and free plasmin was performed by quantifying the amino groups released during β-CN proteolysis, according to the method of Church *et al* (1985), using 2,4,6-trinitrobenzene sulfonic acid (TNBS). Free plasmin (0.02 mg), or plasmin-Sepharose conjugate containing 0.16 mg of immobilized active plasmin, was mixed with six amounts (from 0.5 to 1 mg) of heat-treated β-CN (80°C,10 min) in 1 ml of 0.05 mol/l tetraborate buffer (pH 8.4). Four identical assays were performed for each concentration of β-CN. After incubation at 37°C for 0 to 25 min, the reaction was stopped by adding (88.5 µg/ml) soybean trypsin inhibitor (Merck, Darmstadt, Germany) for free plasmin, or conjugate was removed by centrifugation, for immobilized plasmin. Reaction mixtures or supernatants (0.2 ml) were diluted in 0.9 ml of 0.05 tetraborate buffer (pH 8.4), containing SDS (0.1%), and heated at 100°C for 5 min. After cooling, 0.4 ml of TNBS fresh solution (0.1% in tetraborate buffer) was added. The mixtures were incubated for 30 min at room temperature in complete darkness before measurement of their optical density at 420 nm. Kinetic parameters (V_{max}, maximum velocity of reaction and K_m, Michaelis constant) of the reactivity of free plasmin and plasmin-Sepharose conjugate with β-CN were determined by Lineweaver-Burk reciprocal plot (Lineweaver and Burk, 1934).

Proteolysis of β-CN by free plasmin and plasmin-Sepharose conjugate

Following the procedure of Eigel (1977), the proteolysis (37°C, from 60 to 300 min) of β-CN was

carried out by mixing heat-treated β-CN (80°C, 10 min) with plasmin-Sepharose conjugate or free plasmin, in 0.05 mol/l tetraborate buffer (pH 8.4). Three ratios (w/w) plasmin/β-CN were assayed: 1/37, 1/9 and 1/5 (0.27, 1.12 and 1.93 mg/ml of active plasmin for 10 mg/ml of β-CN, respectively). Polyacrylamide gel electrophoresis in 4.5 mol/l urea, 0.38 mol/l Tris-HCl buffer (pH 8.9) (Ng-Kwai-Hang and Kroeker, 1984) were performed to determine β-CN fragments in reaction supernatants (centrifugation 2500 *g*, 10 min, when plasmin-Sepharose conjugate was used) and in reaction mixture (after adding of soybean trypsin inhibitor, 0.088 mg/ml, for free plasmin).

The influence of β-CN fragments on the proteolysis of β-CN (3.7 mg/ml) by the plasmin-Sepharose conjugate and by free plasmin (0.1 mg/ml of active plasmin) was compared by measuring the residual concentration of β-CN in reaction supernatants (plasmin-Sepharose conjugate) or reaction mixture (free plasmin) after adding of graded concentrations (0.00, 0.05, 0.15, 0.25, and 0.40 mg/ml) of a mixture of C-terminal peptides (β-CN f(29–209), f(106–209) and f(108–209)), or a preparation of purified β-CN f(1–105/7).

β-CN quantification

The quantification of residual β-CN after proteolysis was performed by microparticle-enhanced nephelometric immunoassay (Montagne *et al*, 1991). Hydrophilic and polyfunctional microspheres (mean dry diameter = 220 nm, SD = 7 nm, $n = 49$), produced as previously described (Marchand *et al*, 1992) and coated with β-CN (MS-β-CN conjugate), were agglutinated by anti-native β-CN antiserum (El Bari *et al*, 1991). Free β-CN, inhibiting this microparticle agglutination, was quantified by the measurement of the light scattered during the agglutination inhibition. Supernatants of β-CN proteolysis (50-fold diluted) were mixed with MS-β-CN conjugate (50 mg/l) and anti-native β-CN antiserum (300-fold diluted) in a 0.05 mol/l borate buffer (pH 8.0) containing 1.5 mmol/l disodium salt of ethylenediaminetetraacetic acid, 30 mmol/l NaN_3, Triton X-100 2 g/l and 30 g/l of polyethylene glycol 6000 (milk nephelometry buffer). The light scattering was measured with the Diagnostics Pasteur (Marnes, France) nephelometer (Nephelia N 600) after 2 h at room temperature. The concentration of β-CN was determined according to a calibration curve performed as previously reported (El Bari *et al*, 1991).

RESULTS

Characterization of protein preparations

The amino-acid composition of the preparations, compared to the composition calculated according to the amino acid sequence of β-CN (Ribadeau-Dumas *et al*, 1972), assessed the presence of β-CN and β-CN f(1–105/7). Electrophoretic patterns of protein preparations on polyacrylamide-urea gel showed the purity of β-CN and β-CN f(1–105/7) reaching 95% and 80%, respectively, and the absence of perceptible contamination of the mixture of β-CN C-terminal peptides by whole β-CN and β-CN N-terminal peptides.

Characterization of the plasmin-Sepharose conjugate

As described in *Materials and methods*, plasmin was dialyzed against 0.1 mol/l carbonate buffer (pH 8) containing 0.5 mol/l NaCl, before its binding on activated Sepharose, to eliminate the great amount of lysine contained in commercial preparation. A strong decrease (60%) of the proteolytic activity of plasmin for the chromogenic substrate D-valyl-L-leucyl-L-lysyl-4-nitroanilide was observed during this dialysis (36 h at 4°C). However, a high binding yield and a good preservation of the reactivity of the immobilized plasmin for the chromogenic substrate were observed. The activity of the immobilized plasmin in plasmin-Sepharose conjugate reached 95% of the activity of the dialyzed plasmin used for binding. No reaction with the chromogenic substrate was measurable in the binding supernatant, and 5.7 mg of active plasmin

were immobilized per g of gel. Plasmin-Sepharose conjugate was stable 6 months at least when it was stored at 4°C in 0.1 mol/l carbonate buffer (pH 8), containing 0.2% NaN_3 as a preservative. Moreover, the conjugate activity for the chromogenic substrate was not significantly altered (P = 0.05) after three successive utilizations.

The kinetic study of β-CN proteolysis, performed by quantifying the amino groups released during plasmin action, permitted to determine the overall velocity of the reaction for six concentrations of β-CN. Kinetic parameters were estimated by reciprocal Lineweaver-Burk plotting of these velocities (fig 1). For immobilized plasmin, V_{max} and

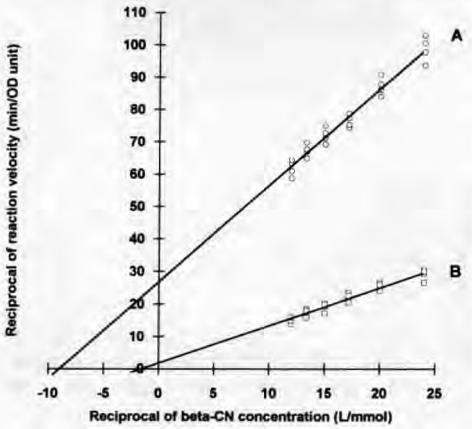

Fig 1. Lineweaver-Burk reciprocal plot for the determination of kinetic parameters of the proteolysis of heat-treated β-CN in 0.05 mol/l tetraborate buffer pH 8.4, by (**A**) plasmin-Sepharose conjugate (n = 24, r = 0.98, P < 0.001, slope = 3.03, V_{max} = 3.83 10^{-2} OD unit/min, K_m = 1.2 10^{-4} mol/l) and (**B**) free plasmin (n = 24, r = 0.97, P < 0.001, slope = 1.16, V_{max} = 53.5 10^{-2} OD unit/min, K_m = 6.2 10^{-4} mol/l). *Détermination, par représentation de Lineweaver-Burk, des paramètres cinétiques de la protéolyse de la β-CN en borate 0.05 mol/l pH 8.4 par :* (**A**) *le conjugué plasmine-sepharose* (n = 24, r = 0,98; P < 0,001; *pente* = 3,03; V_{max} = 3,83 10^{-2} *unité DO/min;* K_m = 1,2 10^{-4} *mol/l) et* (**B**) *la plasmine libre* (n = 24, r = 0,97; P < 0,001; *pente* = 1,16; V_{max} = 53,5 10^{-2} *unité DO/min;* K_m = 6,2 10^{-4} *mol/l).*

K_m (3.8 10^{-2} OD unit/min and 1.2 10^{-4} mol/l, respectively) were less than that found for free plasmin (53.5 10^{-2} OD unit/min and 6.2 10^{-4} mol/l).

Comparison between the proteolysis of β-CN by free and immobilized plasmin

Early appearance of fragments of β-CN in polyacrylamide-urea gel electrophoresis (Eigel, 1977) showed the rapid proteolysis of β-CN by free plasmin (fig 2A). The evolution of the relative concentrations of β-CN, β-CN f(1-105/7) and β-CN f(29-105/7) as a function of the proteolysis time is shown in figure 2B. As early as at 30 min of proteo_lysis by free plasmin, residual native β-CN represented only 9% of the proteins contained in the proteolysis mixture and its relative concentration continued to decrease, reaching 1% after 200 min of reaction. The concentration of the fragment 1–105/7 of β-CN rapidly increased (18% of proteins at 30 min), before a decrease so large as to disappear. The increase of the relative concentration of fragment 29–105/7 was more slow: 19% after 100 min of proteolysis, when the concentration of β-CN f(1–105/7) was already low (8% of the total proteins in reaction mixture).

In comparison with the previous results obtained for free plasmin, figure 3 shows the electrophoretic patterns of reaction supernatants (fig 3A) and the evolution of their relative concentrations in β-CN, β-CN f(1–105/7) and β-CN f(29–105/7) (fig 3B) during the proteolysis of β-CN by the plasmin-Sepharose conjugate. Appearance of several bands in polyacrylamide-urea gel electrophoresis confirmed the hydrolysis of the β-CN molecule by the conjugate. This reaction was slower than that observed with free plasmin (relative concentration of native β-CN in the proteolysis supernatant was 38% after 60 min of reaction) and it was still

incomplete after 300 min (native β-CN constituted 15% of the proteins). An increase of the ratio active plasmin/β-CN from 1/37 to 1/9 and then 1/5 (data not shown) produced an increase of the rate of reaction (22% of residual β-CN only at 60 min of proteolysis) but did not change the percentage of residual native β-CN in proteolysis supernatant (15%) after 300 min of reaction.

The residual concentration of native β-CN, after β-CN proteolysis (3.7 mg/ml, 0.1 mg/ml of active plasmin, 37°C, pH 8.4,

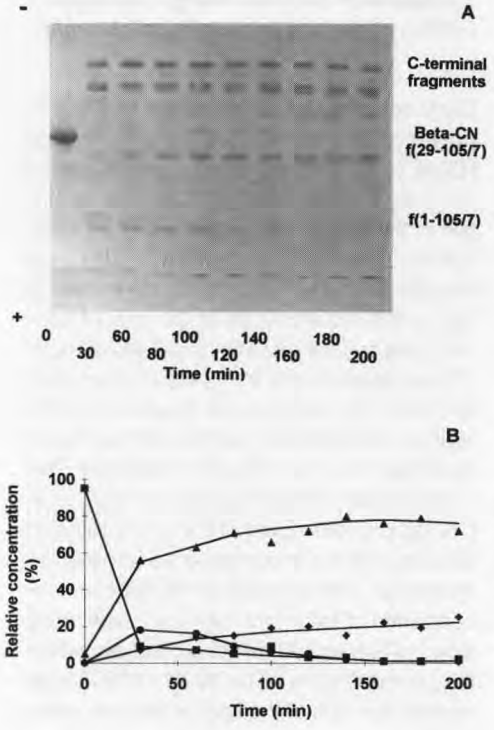

Fig 2. Proteolysis (37°C, pH 8.4) of β-CN (10 mg/ml) by free plasmin (0.27 mg/ml). **A.** Electrophoretic patterns of reaction mixtures on polyacrylamide gel in pH 8.9 urea buffer. **B.** Evolution, determined by densitometric analysis, of the relative concentrations of β-CN (■), β-CN f(1–105/7) (●), β-CN f(29–105/7) (◆), and other fragments of β-CN (▲), as a function of the proteolysis time.

Protéolyse (37°C, pH 8,4) de la β-CN (10 mg/ml) par la plasmine libre (0,27 mg/ml). **A.** *Étude électrophorétique des milieux de réaction, sur gel de polyacrylamide en tampon urée pH 8,9.* **B.** *Évolution, en fonction de la durée de la protéolyse, des concentrations relatives de β-CN* (■), *β-CN f(1–105/7)* (●), *β-CN f(29–105/7)* (◆), *et des autres fragments de la β-CN* (▲).

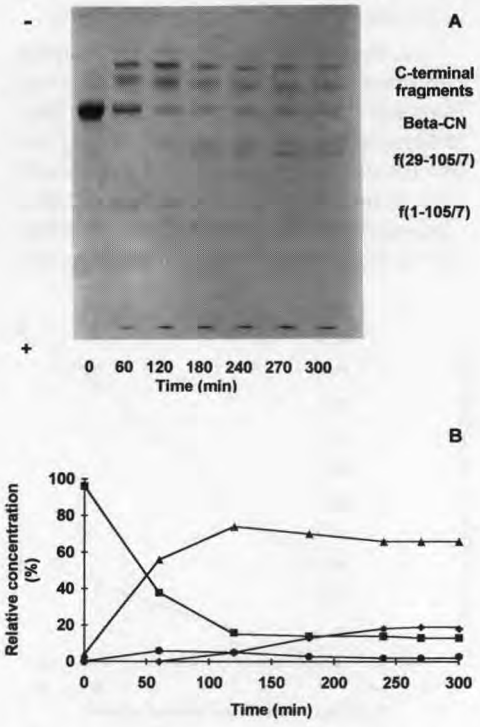

Fig 3. Proteolysis (37°C, pH 8.4) of β-CN (10 mg/ml) by plasmin-Sepharose conjugate (0.27 mg/ml of active plasmin). **A.** Electrophoretic patterns of reaction supernatants on polyacrylamide gel in pH 8.9 urea buffer. **B.** Evolution, determined by densitometric analysis, of the relative concentrations of β-CN (■), β-CN f(1–105/7) (●), β-CN f(29–105/7) (◆), and other fragments of β-CN (▲), as a function of the proteolysis time.

Protéolyse (37°C, pH 8,4) de la β-CN (10 mg/ml) par le conjugué plasmine-sepharose (0,27 mg/ml de plasmine active). **A.** *Étude électrophorétique des milieux de réaction, sur gel de polyacrylamide en tampon urée pH 8,9.* **B.** *Évolution, en fonction de la durée de la protéolyse, des concentrations relatives de β-CN* (■), *β-CN f(1–105/7)* (●), *β-CN f(29–105/7)* (◆), *et des autres fragments de la β-CN* (▲).

2 h) by plasmin-Sepharose conjugate and free plasmin, was determined by microparticle-enhanced nephelometric immunoassay (table I). Residual β-CN concentrations of 0.35 and 0.15 mg/ml (about 10 and 4% of the original concentration) were found in the reaction supernatants when immobilized and free plasmin were respectively used. These residual β-CN concentrations were not significantly changed when β-CN fragments (mixture of C-terminal peptides and purified β-CN f(1–105/7)) were added in the proteolysis mixture, from 0.05 to 0.40 mg/ml.

DISCUSSION

Activated CH-Sepharose 4B, retained as a solid phase to immobilize bovine plasmin, was constituted by an agarose matrix bearing carboxyl-activated functions anchored to the gel through a spacer arm six carbon atoms long (Cuatrecasas, 1970). Primary amino groups of plasmin reacted in smooth conditions with formation of stable amide bonds and release of N-hydroxysuccinimide. Reaction of ε-amino groups of plasmin was favoured by the basic pH (carbonate buffer pH 8) chosen for the binding. The porosity of Sepharose 4B (exclusion limit 20 10^6 Da) permitted a binding of enzyme both on the surface and at the interior of the gel beads and a free diffusion of its substrates into the pores. The spacer arm between the gel and plasmin rendered the immobilized enzyme more accessible.

A high binding yield of plasmin to activated-Sepharose and a good preservation of the proteolytic activity of the immobilized enzyme for the chromogenic substrate (95% of the activity of the free plasmin used for the binding) was thus obtained in the plasmin-Sepharose conjugate (5.7 mg of active plasmin per g of gel). However, a strong decrease (60%) of the proteolytic activity of plasmin for the chromogenic substrate was

Table I. Residual β-CN after proteolysis [a], by plasmin-Sepharose conjugate and free plasmin, in the presence of β-CN fragments.
β-CN résiduelle après protéolyse [a], par le conjugué plasmine-sepharose et la plasmine libre, réalisée en présence de fragments de la β-CN.

	Concentration, mg/ml of β-CN fragments	*Concentration, mg/ml (% [b]) of residual β-CN after proteolysis by*			
		Immobilized plasmin		*Free plasmin*	
	0.00	0.35	(9.5)	0.15	(4.1)
β-CN f(1-105/7)	0.05	0.38	(10.3)	0.20	(5.4)
	0.15	0.32	(8.6)	0.15	(4.0)
	0.25	0.35	(9.4)	0.24	(6.5)
	0.40	0.33	(8.9)	0.24	(6.5)
Mixture of	0.05	0.27	(7.3)	0.14	(3.8)
β-CN f(29–209)	0.15	0.26	(7.0)	0.16	(4.3)
and	0.25	0.25	(6.8)	0.15	(4.1)
f(106/8–209)	0.40	0.30	(8.1)	0.17	(4.6)

[a] β-CN: 3.7 mg/ml; active plasmin: 0.1 mg/ml; 37°C; pH 8.4; 2 h. [b] Percentage of the original concentration.
[a] β-CN: 3,7 mg/ml; plasmine active: 0,1 mg/ml; 37°C; pH 8,4; 2 h. [b] Pourcentage de concentration première.

observed during dialysis, performed before immobilization to eliminate the lysine contained in commercial preparation. Such a decrease of the proteolytic activity during dialysis, already observed (Humbert, 1986), might be due to a partial autolysis of the free plasmin molecule at neutral or basic pH (Walsh and Wilcox, 1970). On account of the stability of the plasmin-Sepharose conjugate further observed, immobilized enzyme appeared protected against this denaturation.

Kinetic study of β-CN proteolysis indicated a perceptible loss of enzyme reactivity for β-CN in plasmin-Sepharose conjugate compared to free plasmin. This difference of reactivity might be the consequence of the molecular mass of β-CN (24 000 Da), higher than that of the chromogenic substrate used (479 Da), reducing the accessibility of the immobilized plasmin to β-CN by steric hindrance at the solid-liquid interface.

The comparative electrophoretic study performed during β-CN proteolysis by free and immobilized plasmin showed that β-CN appears incompletely hydrolyzed by plasmin-Sepharose conjugate in conditions of full reaction with free plasmin. The proteolysis of β-CN f(1–105/7) by plasmin, producing β-CN f(29–105/7) especially (Eigel, 1977; Andrews and Alichanidis, 1983), was observed both with immobilized and free plasmin. The evolution of the relative concentrations of β-CN, β-CN f(1–105/7) and β-CN f(29–105/7), during the proteolysis of β-CN by plasmin, probably indicated a competitive effect of the peptide β-CN f(1–105/7) on the reaction of plasmin with whole β-CN. The assay of residual β-CN in supernatants of proteolysis was used to determine this effect.

The microparticle-enhanced nephelometric immunoassay of β-CN previously reported by El Bari *et al* (1991) is suitable to assay β-CN in the presence of the β-CN fragments formed during the β-CN proteolysis by plasmin because of the slight cross reactivity of these β-CN fragments with antinative β-CN antisera: 0.04% and 1.4% for β-CN f(1–105/7) and β-CN f(106–209) compared with native β-CN, respectively (El Bari *et al*, 1992). Microparticle-enhanced nephelometric immunoassay of β-CN could thus be used even after adding of graded concentrations of β-CN fragments. The residual β-CN concentration in supernatants of β-CN proteolysis was higher with immobilized (about 10%) than with free plasmin (about 4%). But, for both immobilized and free plasmin, no significant influence of β-CN fragments (β-CN f(1–105/7) or mixture of C-terminal peptides (β-CN f(29–209), f(106–209) and f(108–209)) was detected on the residual β-CN concentration.

The immobilization of bovine plasmin on a solid phase such as Sepharose beads thus permitted to obtain a reactive conjugate. Compared with free enzyme, kinetics of enzyme action was only delayed. Changes of reactivity of immobilized proteases have been previously reported. They were generally imputed to a decrease of enzyme charge (Reimerdes, 1979) produced by consumption of amino or carboxyl groups during the immobilization process and inducing a stronger adsorption of hydrophobic components, a modification of the optimum conditions of enzyme activity and finally, a decrease of the catalytic activity (Gabel and Hofsten, 1970). Such alterations of enzyme charge and of its ionic surroundings at solid-liquid interface might be the cause of the loss of reactivity of plasmin with β-CN, observed in plasmin-Sepharose conjugate.

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