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INTERACTION OF IMMOBILIZED HUMAN PLASMINOGEN AND PLASMIN WITH STREPTOKINASE

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The complex of immobilized human plasminogen (iHPlg) and streptokinase (SK) has a lower catalytic activity in hydrolysis of plasmin-specific substrate S-2251 with a k_{cat}/K_m of 0.005 $\mu M^{-1} \cdot s^{-1}$, compared to 0.031 $\mu M^{-1} \cdot s^{-1}$ of immobilized human plasmin (iHPlm) and 0.078 $\mu M^{-1} \cdot s^{-1}$ of HPlm. The SK in the complex could be removed by acid buffer solution, and the iHPlg will remain catalytically active. IHPlm·SK complex could activate bovine plasminogen (BPlg), whereas iHPlg·SK complex could not. IHPlg could be activated by interaction with a combination of SK and HPlg or urokinase. The activated iHPlg·SK complex had the ability to activate BPlg as iHPlm·SK. The reasonable explanation is that iHPlg was converted to iHPlm in reaction with the combination of HPlg and SK. However, iHPlg was converted to a virgin enzyme in reaction with SK alone and could not activate BPlg. A new modified mechanism for the interaction of HPlg or HPlm with SK was proposed.

Activation of mammalian plasminogen (Plg) to plasmin (Plm), which is the major fibrinolytic enzyme, occurs as a result of cleavage of a peptide bond at Arg^{560}-Val^{561} in the zymogen molecule (1-3). Several Plg activators, such as streptokinase (SK), urokinase and tissue-type plasminogen activator can catalyze the reaction and are clinically used as thrombolytic agents (4). The bacterial Plg activator SK is unique in that it is a proactivator with no proteolytic activity (5, 6). The true activator is formed as it interacts with some species-specific Plg or Plm (7-11). The interaction between protease active site domain of Plg or Plm and SK could be the main cause for the complex formation (12-16). Previous studies have demonstrated that human plasminogen (HPlg) or human plasmin (HPlm) and SK equimolar complex can activate Plg of other species, such as bovine plasminogen (BPlg), which cannot be activated by SK alone (12, 17-19). The HPlg·SK complex was suggested to be an efficient Plg activator (8, 20). Binding

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Abbreviations used: Plg, plasminogen; Plm, plasmin; HPlg and BPlg, human and bovine plasminogens, respectively; HPlm and BPlm, human and bovine plasmins, respectively; iHPlg and iHPlm, immobilized human plasminogen and plasmin, respectively; SK, streptokinase, and vHPlg, virgin enzyme of human plasminogen.

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of SK to HPlg was found to induce the formation of a functional activator active site in the HPlg moiety (7, 8, 19). As a result, a virgin enzyme (vHPlg), a catalytically active form of HPlg with intact Arg^{560}-Val^{561} peptide bond, was detected as SK was separated from the HPlg·SK complex by acid solution (21).

It has been suggested that HPlg·SK can be converted to HPlm·SK intramolecularly, although no direct evidence has been provided (21-24). In this study, iHPlg and iHPlm were used to reinvestigate the mechanism of interaction between SK and Plg or Plm. The iHPlg or iHPlm would limit the interaction between molecules of Plg or Plm. The linkage with the solid support would prevent the reactions that require the intermediates containing two HPlg or HPlm, such as HPlg·SK·HPlg or HPlm·SK·HPlm. However, the formation of these intermediates becomes possible if HPlg or HPlm is added to the solution. Therefore, iHPlg was applied to study the conversion of the HPlg molecule in the HPlg·SK complex to a virgin enzyme or to an HPlm. The amidolytic activity and the ability to activate BPlg of these complexes were also studied.

MATERIALS AND METHODS

Proteins and enzymes: HPlg was prepared from pooled human plasma by a modification of the Deutsch and Mertz method (25). Forms 1 and 2 of native human Glu-Plg were separated by chromatography on Lys-Sepharose column (26). Form 2 of Plg was used throughout the experiment. HPlm was prepared by activating HPlg with Sepharose-bound urokinase as previously described (15). BPlg was purified from fresh citrated bovine plasma by the same affinity chromatography method. SK (750,000 IU/mg, from Behringwerke AG, Marburg, W. Germany) was further purified by passing it through a blue-Sepharose CL 6B column (0.9 x 40 cm) to remove serum albumin (17).

Preparation of protein substituted Sepharose: HPlg or HPlm (6 mg) was coupled to CNBr-activated Sepharose 4B (1 g, from Pharmacia LKB Biotechnology Inc.) in a coupling buffer containing 0.1 M $NaHCO_3$, 0.5 M NaCl (pH 8.3) at 4°C overnight. The gel was incubated and washed with 1 M ethanolamine in 0.1 M $NaHCO_3$, 0.5 M NaCl (pH 8.0) to eliminate the residual active groups. The gel was then washed with coupling buffer (pH 8.3) and 0.1 M acetate buffer, 0.5 M NaCl (pH 4.0) alternately and was preserved in activating buffer pH 6.5, containing 0.05 M phosphate, 0.1 M NaCl, 0.02 M lysine, and 25% glycerol.

Protein and enzyme concentration: Protein concentrations were determined spectrophotometrically using the following $\varepsilon^{1\%}$, 280 nm values and molecular weights, respectively: HPlg and BPlg, 17.0 and 84,000 (2, 27, 28); HPlm, 17.0 and 76,500 (28); SK, 9.5 and 45,000 (29). Active site concentration of HPlm was determined by the *p*-nitrophenyl-*p*'-guanidinobenzoate burst titration (15, 30). The active concentration of iHPlm was determined by measuring its amidolytic activity according to a standard HPlm calibration curve. The amount of protein on the immobilized gel was determined by amino acid analysis. IHPlg or iHPlm (0.1 g) was washed extensively with 30 ml of distilled water, vacuum-dried, and hydrolyzed in 6 N HCl (Sequanal-grade, Pierce Chemical Co.) at 110°C for 24 h. Amino acid analysis was performed on a Beckman Model 6300 amino acid analyzer.

Amidase parameter measurement: The enzymatic activities of iHPlm and iHPlg·SK complex were measured with the peptide substrate, NH_2-D-Val-Leu-Lys-*p*-nitroanilide (S-2251), at 37°C and in 0.05 M Tris-HCl, pH 7.4, 0.1 M NaCl. The substrate concentration varied between 0.2 and 4 K_m. At the indicated time, the reaction mixture was rapidly filtered through a plastic syringe with a tight glass-wool plug at the end. The absorbance at 405 nm of the filtrate was measured. The initial rate and substrate concentration data were analyzed on a Lineweaver-Burk plot. The ε^{1M} at 405 nm employed for *p*-nitroanilide was 9559.

Preparation of iHPlm·SK complex, iHPlg·SK complex, and virgin enzyme (vHPlg): IHPlm (4 μM) or iHPlg (4 μM) was interacted with equimolar SK in 0.5 ml activating buffer (pH 6.5) at 25°C for 30 min with continuous stirring. The iHPlg·SK complex was an amidolytically active virgin enzyme. The iHPlg·SK or iHPlm·SK complex was packed into a small plastic column and washed with 20 ml of activating buffer (pH 6.5) followed by 20 ml of 0.05 M phosphate, 0.1 M NaCl, 0.02 M lysine, 25% glycerol buffer (pH 2.2) at 4°C to remove SK. Protein labeled with ^{125}I was used to monitor the removal of SK and HPlg added in the solution. The gels were resuspended in 0.5 ml of activating buffer for enzyme activity assay.

^{125}I-labeling of protein: SK or HPlg (100 μl) at a concentration of 1.0 mg/ml was incubated with $Na^{125}I$ (100 μCi) and slightly agitated at room temperature in an eppendorf tube previously coated with 100 μl Iodo-gen (0.08 mg/ml) (Sigma) for 8 min. After incubation, the reaction mixture was applied on a PD-10 column (Pharmacia, Sweden) and eluted with 0.01 M phosphate buffer (pH 7.2). The labeled protein was collected after discarding the void volume.

Amidolytic activity measurement: To measure the amidolytic activity of the immobilized enzyme, 30 μl of the gel suspension (containing immobilized enzymes at a final concentration of 0.24 μM) was added into 0.5 ml of 0.05 M Tris-HCl (pH 7.4) buffer containing 0.5 mM peptide substrate S-2251 and 0.1 M NaCl at 37°C. The rate increment of absorbance at 405 nm of the supernatant was recorded.

BPlg activator activity measurement: BPlg (5 μM) was incubated with various preparations of iHPlm·SK or iHPlg·SK in 0.5 ml of activating buffer at 37°C. At the time indicated, 50 μl aliquot of the reaction mixture was pipetted to a cuvette containing 0.5 mM peptide substrate (S-2251) in 0.05 M Tris-HCl (pH 7.4), 0.1 M NaCl. The initial rate of increment of absorbance at 405 nm was recorded. The reaction without BPlg was used as control.

Activation of iHPlg: IHPlg before and after washing with 20 ml of cold 0.05 M phosphate, 0.1 M NaCl, 0.02 M lysine, 25% glycerol buffer (pH 2.2) was incubated with catalytic amount of urokinase, SK, or equimolar HPlm·SK in activating buffer (pH 6.5) at 30°C. At the time indicated, 25 μl of the gel suspension was added to 0.5 ml of 0.05 M Tris-HCl (pH 7.4) containing 0.5 mM S-2251 and 0.1 M NaCl at 37°C and the absorbance at 405 nm of the supernatant was recorded. The initial rate was calculated.

SDS-polyacrylamide gel electrophoresis: The basic techniques of gel electrophoresis were done as described by Weber and Osborn (31).

Reagents: S-2251, urokinase, and *p*-nitrophenyl-*p*'-guanidinobenzoate were obtained from Sigma. Blue-Sepharose CL 6B, Sepharose 4B, and CNBr-activated Sepharose 4B were purchased from Pharmacia LKB Biotechnology, Inc. All other chemicals used were of the highest grade commercially available.

RESULTS

HPlg and HPlm immobilized onto CNBr-activated Sepharose 4B gel were used to study the interaction with SK. IHPlg became amidolytically active as equal molar of SK was added to the solution (Fig. 1). The SK was separated from the gel of iHPlg (>97%) by washing with activating buffer followed by acid solution as monitored with ^{125}I-labeled SK (Table 1). When the SK moiety had been separated from the iHPlg·SK, the gel retained about 30% of amidolytic activity (Fig. 1). The amidase activities of the iHPlg·SK complex, acid-washed iHPlg·SK complex, and the iHPlm are summarized in Table 2. The K_m of iHPlm (746 μM) was about twice that of the soluble HPlm (355 μM). The k_{cat} of iHPlm and HPlm were similar (23.3 and 27.9 s^{-1}, respectively). However, iHPlg·SK complex had lower catalytic activity. The k_{cat}/K_m of the complex was lower than that of iHPlm (Table 2). The acid-washed iHPlg·SK had similar K_m but a smaller k_{cat} compared to that of iHPlg·SK.

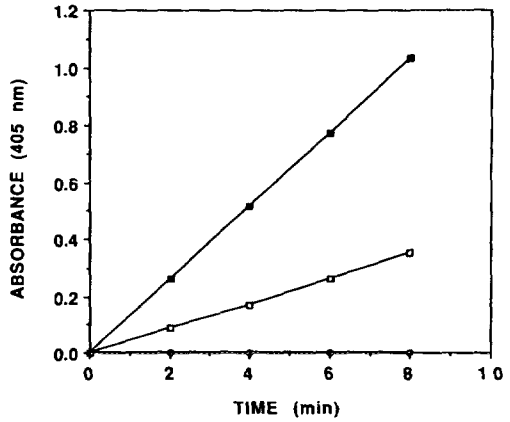

Fig. 1. Amidolytic activity of the virgin enzyme derived from the iHPlg. The amidolytic activities of 0.24 μM iHPlg (•), iHPlg·SK before (■) and after (□) washing extensively with acid buffer are shown.

The iHPlm·SK complex was amidolytically active and could activate BPlg (Fig. 2A and 2B). After separation of SK from iHPlm·SK by acid buffer, the immobilized gel lost the ability to activate BPlg but retained its amidolytic activity (Fig. 2A and 2B). In contrast, iHPlg·SK complex was amidolytically active but could not activate BPlg (Fig. 3A and 3B). The iHPlg, after incubating with equal molar SK and soluble HPlg, exhibited BPlg activator activity in the presence of SK (Fig. 3B). The covalent binding of protein to Sepharose gel was stable since the amount of protein on the gel after treatment with cold acid buffer did not significantly decrease as measured by amino acid analysis (Table 3). The acid buffer treatment did not cause denaturation of HPlm or HPlg since more than 90% of the amidolytic activity of iHPlm was recovered after the acid wash. IHPlg was functionally active before and after acid wash as it could be activated by urokinase or equimolar HPlm·SK complex (Fig. 4). In reaction with equimolar iHPlm, SK was cleaved and major peptide fragments of 40 KDa, 36 KDa, 34 KDa and 30 KDa were found in the reaction mixture at 10 min incubation as shown by SDS gel electrophoresis. After 30 min, SK was further cleaved into peptide fragments of 30 KDa and 18 KDa. However, in reaction with iHPlg, no

Table1. Interaction of iHPlm and iHPlg with ^{125}I-SK and soluble ^{125}I-HPlg

Contents of the reaction mixture	% of total radioactivity		
	pH 6.5 eluent	pH 2.2 eluent	immobilized gel
iHPlm + SK*	91.5	6.9	1.6
iHPlg + SK*	86.9	10.5	2.6
iHPlg + SK* + HPlg	97.9	1.3	0.8
iHPlg + SK + HPlg*	96.0	1.8	2.2

IHPlm (4 μM) or iHPlg (4 μM) was incubated with equimolar ^{125}I-SK (SK*) in 0.5ml of 0.05 M phosphate, 0.1 M NaCl, 0.02 M lysine, 25% glycerol buffer (pH 6.5) at 25°C for 30 min and then treated with buffer alone, or soluble unlabeled or labeled HPlg (4 μM) for 30 min. The immobilized gel was washed extensively with 20 ml of pH 6.5 buffer and then 20 ml of pH 2.2 buffer at 4°C. Eluents and the remaining immobilized gel were collected to measure the radioactivities with a LKB γ-counter.

Table 2. Amidase parameters of HPlm, iHPlm, and iHPlg·SK complex with S-2251 at pH 7.4 and 37°C

Enzyme species	Amidase parameters		
	K_m, μM	k_{cat}, s^{-1}	k_{cat}/K_m, $\mu M^{-1}.s^{-1}$
HPlm	355 ± 3	27.9 ± 0.3	0.078
iHPlm	746 ± 8	23.3 ± 0.8	0.031
iHPlg·SK	1151 ± 15	5.7 ± 0.6	0.005
Acid-washed iHPlg·SK	1052 ± 33	1.9 ± 0.1	0.0018

Values given are means ± S.E. (n=4).

peptide bond cleavage of SK was observed, and only intact SK molecule was detected in the reaction mixture (Fig. 5).

DISCUSSION

SK has been used as a thrombolytic agent for decades because of its specific interaction with HPlg or HPlm to form a complex which can activate Plg from different species. In the HPlg and SK reaction, an equimolar complex was detected (7, 8, 10, 32). The complex was rapidly converted to HPlm·SK' (modified SK) (22-24). It was also suggested that HPlg·SK was a more effective activator than HPlm·SK (8, 20). In the interaction of HPlg and SK, a virgin enzyme was observed (24). However, in the BPlg activation process catalyzed by HPlg·SK complex, the complex was quickly converted to HPlm·SK' (22-24). Therefore, it was difficult to conclude which form of HPlg and SK complex was the major activator that catalyzed the conversion of BPlg to BPlm. It was also suggested that HPlg·SK could be intramolecularly converted to HPlm·SK' (21-23). However, no unequivocal evidence was provided to support whether the conversion was a unimolecular reaction or was catalyzed by second HPlg or HPlm molecule.

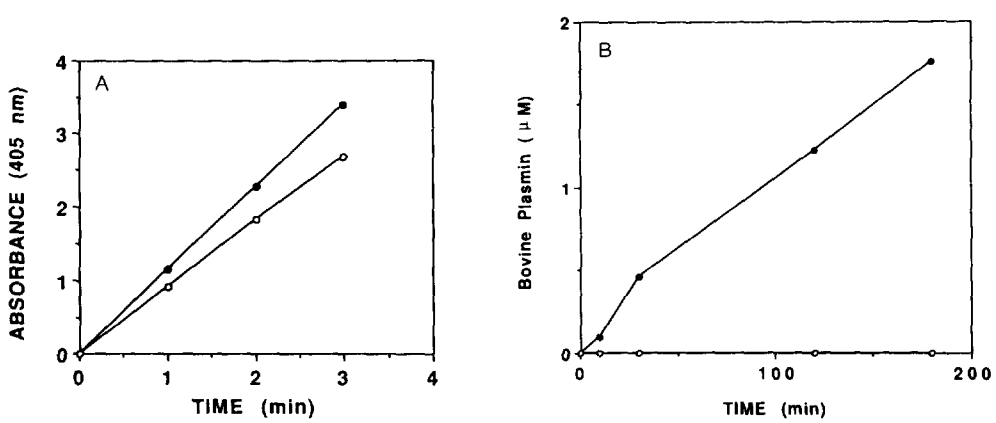

Fig. 2. The amidolytic activity (A) and BPlg activator activity (B) of iHPlm·SK complex and the effect of washing with acid buffer. The amidolytic activity was assayed by mixing substrate S-2251 (0.5 mM) with equimolar complex of iHPlm and SK (0.24 μM), before (•) and after (○) washing with acid buffer. The same gel samples (0.24 μM) were assayed for BPlg activator activity by mixing the gel suspension with BPlg (5 μM); the solution was assayed for BPlm produced at determined time periods based on a BPlm calibration curve.

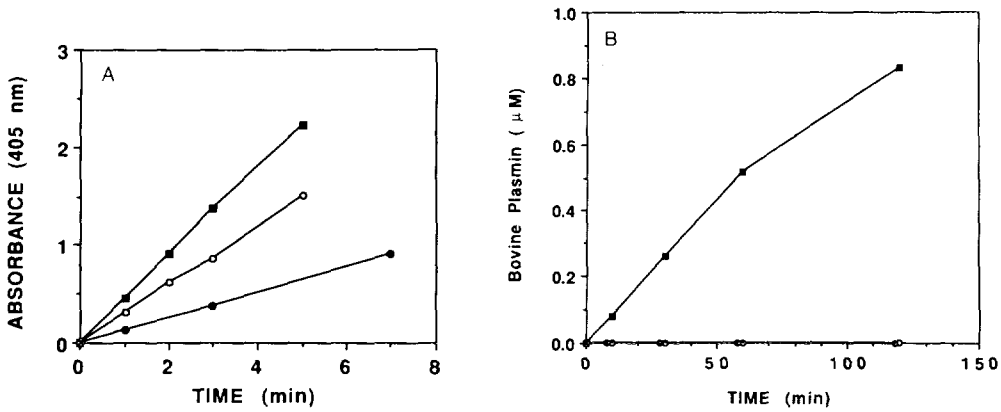

Fig. 3. The amidolytic activity (A) and BPlg activator activity (B) of the iHPlg derivatives. IHPlg (4 μM) was incubated with SK (4 μM) and soluble HPlg (4 μM) for 30 min at 25°C and washed free of SK and HPlg with cold buffers of pH 6.5 and 2.2, subsequently. The immobilized gel was then resuspended in 0.5 ml of pH 6.5 activating buffer with SK (4 μM). The amidolytic activity (A) and BPlg activator activity (B) of iHPlg·SK (•), iHPlg after interaction with SK and HPlg and after washing (○), and the mixture of SK plus the former HPlg·SK treated iHPlg (■) were measured as in previous figure.

In this study, iHPlg was used to study the conversion of HPlg·SK to virgin enzyme and to HPlm·SK. Interaction of SK with iHPlg gel suspension was limited to the reaction in which only one HPlg molecule was involved, since the interaction among HPlg on the solid support was limited. Therefore, iHPlg and iHPlm was applied to differentiate the reaction either with one or more than one HPlg involved.

The iHPlg proved to be functionally intact since it could be activated by urokinase or HPlm·SK complex, and the activated iHPlg had similar amidolytic activity as iHPlm (Fig. 2A, 3A). The k_{cat} of the activated iHPlg was about half that of iHPlm. This could be due to the steric hindrance that might have interfered with the activation of some of the HPlg molecules on the gel. The iHPlg and iHPlm had similar ability to form complex with SK; 7 to 10% of SK was retained by the gel even after washing with large volume of buffered solution. The complex was unstable and dissociated in pH 2.2 buffer.

IHPlg·SK complex was amidolytically active (Fig. 1). The HPlg on the gel remained amidolytically active as SK moiety was removed. The iHPlg·SK complex was different from the iHPlm·SK in three respects. First, the complex of iHPlg·SK was less active amidolytically than the iHPlm or iHPlm·SK complex. The k_{cat}/K_m was about 1/15 of HPlm (Table 2).

Table 3. Protein amount of iHPlg before and after washing with acid buffer

	Amount of Plg (nmole)
Control	3.04 ± 0.04
Acid-washed	2.87 ± 0.03

Values given are means ± S.E. (n=4)
IHPlg was packed into a small plastic column and washed extensively with 20 ml of cold activating buffer (pH 6.5) followed by 20 ml of cold 0.05 M phosphate, 0.1 M NaCl, 0.02 M lysine, 25% glycerol buffer (pH 2.2). The immobilized gel was then washed with 30 ml of water and amount of protein was determined by amino acid analysis.

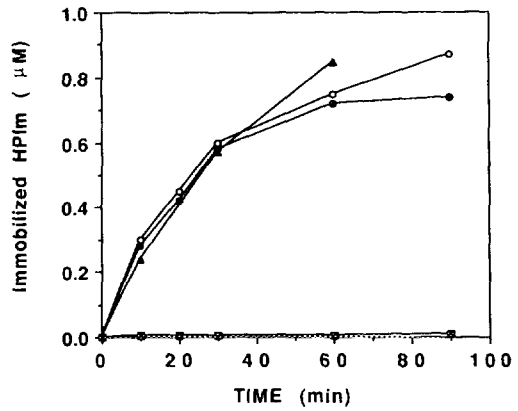

Fig. 4. Activation of iHPlg by urokinase, HPlm·SK, and SK and the effect of washing with acid buffer. Samples of iHPlg (3 μM), before (closed symbol) and after (open symbol) washing with acid phosphate buffer was activated by different activators. Samples of gel suspension were mixed with urokinase (0.06 μM, ●, ○), HPlm·SK (0.06 μM, ▲), and SK (0.06 μM, ■) in activating buffer (pH 6.5) at 30°C. HPlm·SK complex was prepared by premix equimolar concentration of HPlm and SK at 25°C for one min before adding to the reaction mixture. Samples were withdrawn at the times indicated, and amidolytic activity was assayed. The amount of iHPlm was determined on a iHPlm calibration curve.

Second, the SK moiety in interaction with iHPlg remained intact but was cleaved rapidly to fragments in interaction with iHPlm (Fig. 5). Third, the iHPlm·SK but not iHPlg·SK catalyzed the activation of BPlg. Therefore, iHPlg was not converted to iHPlm·SK or iHPlm·SK' in interaction with SK under the experimental conditions. The amidolytically active iHPlg could be a virgin enzyme with intact Arg^{560}-Val^{561} peptide bond which has been characterized in soluble form of HPlg·SK complex (21). When the HPlg as well as SK were added to the iHPlg

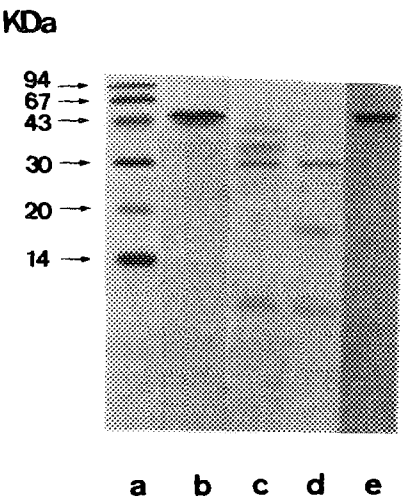

Fig. 5. SDS-gel electrophoresis of the interaction of iHPlm and HPlg with SK. Small portions of the reaction mixture supernatant of iHPlm (4 μM) and SK (4 μM) were taken for SDS gel electrophoresis after incubation at 25°C for various time interval as indicated: 0 (lane a), 10 (lane b), and 30 (lane c) min. Similar incubation of iHPlg with SK for 30 min (lane d) is shown.

suspension, the iHPlg was apparently converted to iHPlm. The activated iHPlg had similar catalytic activity either as amidase or BPlg activator as iHPlm. Therefore, SK alone cannot activate iHPlg probably because the activation of HPlg by SK required second molecule of HPlg or HPlm for the conversion of HPlg to HPlm. The reaction process that took place could be as follows:

$$\text{HPlg} \xrightarrow{\text{SK}} \text{vHPlg·SK} \xrightarrow[\text{or vHPlg·SK}]{\text{HPlg or HPlm}} \begin{matrix}[\text{vHPlg·SK·HPlg}] \\ \text{or} \\ [\text{vHPlg·SK·HPlm}]\end{matrix}$$

$$\longrightarrow \text{HPlm·SK} \longrightarrow \text{HPlm·SK}' \qquad \text{(a)}$$

$$\text{BPlg} \xrightarrow[\text{not by HPlg·SK}]{\text{HPlm·SK or [HPlg·SK·HPlg]}} \text{BPlm} \qquad \text{(b)}$$

In the proposed mechanism, the HPlg·SK complex was converted to the virgin enzyme complex, vHPlg·SK, through conformational rearrangement. The intramolecular activation of vHPlg·SK to HPlm·SK was not observed in this study. The interaction with second molecule of HPlg or HPlm was required to form a trimeric intermediate for the conversion of HPlg to HPlm shown in the reaction scheme (a). The iHPlm·SK can activate BPlg by direct hydrolysis of Arg^{557}-Ile^{558} peptide bond. However, iHPlg·SK could not activate BPlg (b). Kinetics studies of Plg activation by native and mutant HPlg·SK complexes in solution suggested that vHPlg·SK complex in solution was a better activator for BPlg than HPlm·SK complex (8, 20). The discrepancy could be due to the fact that the HPlg and SK complex in solution might have existed as HPlg·SK·HPlg or $(HPlg·SK)_2$ intermediates which could be the effective activator of both BPlg and HPlg. The trimer structure analogue was reported in the mixture of HPlg, SK and HPlm-B chain (24). This kind of complex might not form with iHPlg, SK and BPlg, since no strong interaction of BPlg and SK was observed. Therefore, BPlg could not be activated by interaction with iHPlg·SK. The observation that HPlg·SK complex had higher Plg activator activity than HPlm·SK complex in previous report (8, 20) was probably due to the instability of SK in interaction with HPlm. In reaction with HPlm, the SK moiety was probably hydrolyzed to 30 KDa and 18 KDa fragments that had less Plg activator activity compared to the intact SK (33 and Fig. 5). Further studies to characterize the structure and function of SK and Plg complexes would clarify the mechanism of Plg activation by SK.

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