PLASMINOGEN-FREE PROACTIVATOR B
OBTAINED FROM HUMAN PLASMA

UTAKO OKAMOTO, YUMIKO TAKADA and AKIKAZU TAKADA

Department of Physiology, School of Medicine, Keio University,
Tokyo, Japan

(Received for publication January 20, 1966)

INTRODUCTION

As reported in the previous paper by the authors, two kinds of precursor of plasminogen activator were presented by gel filtration method, which were named proactivator A and proactivator B. However the former fraction did not contain plasminogen, the latter on the other hand always coexisted with plasminogen, which seemed to be difficult to separate. Therefore, a question arises that fibrinolytic activity demonstrated by adding streptokinase (SK) to proactivator B fraction (with plasminogen) might not be mediated by the activation of proactivator but caused directly by the activation of plasminogen by SK.

To resolve the question it is required that proactivator B can be obtained which was separated from plasminogen, and that fibrinolysis should be examined by using thus obtained proactivator B.

The results in this paper are to prove that fibrinolytic activity demonstrated by proactivator B fraction is also mediated by an activator converted from proactivator B as in the case of proactivator A.

MATERIALS AND METHODS

Materials:

1) Dried human plasma (Nihon Seiyaku Co., Ltd., Tokyo) was used as a material of proactivator B fraction obtained by gel filtration.

2) Fibrinogen (Fraction I, Armour Laboratory) and Thrombin (Mochida Co., Ltd., Tokyo) were used to make fibrin plate.

3) Streptokinase (SK, Varidase, Lederle Laboratories) was used to activate
practivator.

4) Urokinase (UK), kindly donated by Green Cross Co., Ltd., Osaka, was used to activate plasminogen.

5) D-aminocaproic acid (EACA) and trans form of aminomethyl cyclo-hexane carboxylic acid (t-AMCHA) were kindly donated by Daiichi Seiyaku Co., Ltd., Tokyo.

6) Proactivator B fraction was separated from dried human plasma by gel filtration using Sephadex G-200 (AB Pharmacia, Uppsala) (see below).

7) Rabbit plasminogen fraction was obtained according to Takada et al.2)

8) Buffers.

As eluant in gel filtration 0.1 M Tris-HCl (pH 8.0) in 0.2 M NaCl was used. To make fibrin plate 0.18 M borate saline buffer (pH 7.75) was used.

Methods:

Gel filtration

Dried human plasma of 400 mg dissolved in 4 ml of Tris-HCl buffer was passed through the Sephadex G-200 column which dimension was 2 x 50 cm. Elution was made with Tris-HCl buffer. Each 3 ml of effluent was taken and proactivator B activity was assayed by clot lysis time, and plasminogen level was assayed by the method of heated fibrin plate as described in our previous papers.1,2)

The effluent which had proactivator B were collected and used as proactivator B fraction in following experiments.

Fibrinolytic assay

Activator activity was measured by Astrup's method3) using standard fibrin plate. Proactivator activity was measured by the same method after activation by SK. Plasminogen activity was measured by the Lassen's method4) using heated fibrin plate after activation of plasminogen by UK or SK.

RESULTS

A) Destruction of plasminogen contained in proactivator B fraction by heating.

Proactivator B fraction was obtained from dried human plasma by the previously mentioned method, and an attempt was made to remove plasminogen activity by heating.

First proactivator B fraction (pH 8.0) obtained by gel filtration was divided into two groups, each of which pH was adjusted at 7.0 and 2.0 respec-
tively by HCl solution. Then each sample was heated at 80°C for 30 minutes in water bath, cooled by running water, and then the pH of the acid sample was readjusted to neutral by adding powdered NaHCO₃. The amount of pro-activator and plasminogen in this heated proactivator B fraction were measured by the following method.

The mixture of 0.1 ml of 500 u/ml SK and 1.0 ml of the heated sample was incubated at 37°C for 10 minutes. Then 0.03 ml of it was placed on a standard fibrin plate and a heated fibrin plate, then incubated at 37°C for 20 hours. The fibrinolytic activity was determined by the lysed area. The result is shown in Table 1.

It was observed in this experiment that proactivator B and plasminogen were both stable at pH 2.0 even heated at 80°C for 30 minutes, however, in pH neutral, plasminogen seemed to lose its activity completely during the process. If proactivator had actually existed in the heated sample at pH 7.0, it was expected that the incubated mixture of this sample, adequate amount of SK and plasminogen would have brought about lysis on the heated plate. Table 2 represents the result of the following investigation. To 1.0 ml of the sample heated at 80°C for 30 minutes at pH 7.0, 0.1 ml of 500 u/ml SK and 1.0 ml of

---

**Table 1**

**Destruction of Plasminogen Included in Proactivator B Fraction by Heating**

<table>
<thead>
<tr>
<th>pH during heating at 80°C for 30 min</th>
<th>Samples added to SK</th>
<th>Lysed area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Standard plate</td>
</tr>
<tr>
<td>2.0</td>
<td>Proactivator B fraction</td>
<td>388</td>
</tr>
<tr>
<td></td>
<td>Heated proactivator B fraction</td>
<td>206</td>
</tr>
<tr>
<td>7.0</td>
<td>Proactivator B fraction</td>
<td>293</td>
</tr>
<tr>
<td></td>
<td>Heated proactivator B fraction</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Physiologic saline</td>
<td>0</td>
</tr>
</tbody>
</table>

was incubated at 37°C for 10 minutes. Then 0.03 ml of it was placed on a standard fibrin plate and a heated fibrin plate, then incubated at 37°C for 20 hours. The fibrinolytic activity was determined by the lysed area. The result is shown in Table 1.

**Table 2**

**Appearance of Fibrinolytic Activity with Heated Proactivator B Fraction after Addition of Rabbit Plasminogen**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Lysed area on heated plate (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heated proactivator B fraction + SK + plasminogen</td>
<td>49</td>
</tr>
<tr>
<td>Heated proactivator B fraction + SK + saline</td>
<td>0</td>
</tr>
</tbody>
</table>
plasminogen-fraction isolated from the rabbit plasma were added, and the mixture was incubated at 37°C for 10 minutes. Saline was used instead of plasminogen as control. In the samples in which plasminogen was added, the lysis on the heated plate was observed in all of the 12 tests but none in the control samples.

B) Examination at different temperatures and periods.

### Table 3

*Preserving of Proactivator after Destroying Plasminogen by Heating in Various Temperatures*

<table>
<thead>
<tr>
<th>Temperature during heating at pH 7.0</th>
<th>Duration for heating (min)</th>
<th>Lysed area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Standard plate</td>
</tr>
<tr>
<td>20°C</td>
<td>120</td>
<td>306</td>
</tr>
<tr>
<td>60°C</td>
<td>30</td>
<td>306</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>300</td>
</tr>
<tr>
<td>80°C</td>
<td>20</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>63</td>
</tr>
<tr>
<td>85°C</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>

Next the proactivator B fraction at pH adjusted to 7.0 was heated for various periods at 60°C, 80°C and 85°C respectively. The results are shown in Table 3.

The Table indicates that heating at 80°C for 20 minutes was sufficient to abolish the plasminogen activity and preserve only proactivator activity.

C) Examination at different pH during heating.

The following experiment was conducted for the purpose of finding the appropriate pH at the time of heating in order to preserve proactivator B and

### Table 4

*Preserving of Proactivator after Destroying Plasminogen by Heating Proactivator B Fraction at 80°C for 20 minutes*

<table>
<thead>
<tr>
<th>Fibrin plate</th>
<th>SK added to 1 ml of samples</th>
<th>pH during heating</th>
<th>Lysed area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0</td>
<td></td>
</tr>
<tr>
<td>Standard plate</td>
<td>20 u.</td>
<td>147 176 178 80 96 81 81 81</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 u.</td>
<td>90 114 125 78 105 68 72 72</td>
<td></td>
</tr>
<tr>
<td>Heated plate</td>
<td>20 u.</td>
<td>57 62 54 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 u.</td>
<td>49 60 81 0 0 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>
Proactivator B fractions at various pH were heated at 80°C for 20 minutes, cooled, and then pH was adjusted at 7.0. Each 0.1 ml of 200 u/ml SK or 100 u/ml SK was added to 1 ml of this sample, and this mixture was incubated at 37°C for 10 minutes in order to activate proactivator B contained in it. The activity of proactivator B and plasminogen was then determined on standard fibrin plate and heated fibrin plate.

As shown in Table 4, at pH 4.0-8.0, the activity of plasminogen was abolished when heated at 80°C for 20 minutes, but that of proactivator was preserved.

D) Investigation in plasminogen destruction by the use of urokinase.

Urokinase (UK) has known to have the ability of converting plasminogen to plasmin. Using this agent plasminogen level in heated proactivator B fraction was examined on heated plate. Proactivator B fraction at pH 8.0 was heated at 80°C for 20 minutes, and 0.1 ml each of UK with various units was added to 1 ml of this sample as shown in Table 5. The existence of plasminogen contained in the sample was examined by the plate method which is the same as employed in the previously mentioned experiment. Table 5 represents the results. The figures in the table show the average of 4 tests.

The results examined with UK indicate that plasminogen was destroyed while proactivator B was preserved under this heating condition, which is the
same result as obtained from the experiment in which SK was used.

This Table reveals that the size of the lysed area on the standard plate caused by the UK added to the heated sample was always bigger than that demonstrated when the same amount of UK was added to physiologic saline. This fact implies that UK had, not only plasminogen-activating effect which it had been said to have, but also the effect of converting proactivator to activator.

E) Additive action of proactivator B.

Twenty ml of proactivator B fraction at pH 8.0 was heated at 80°C for 15 minutes and lyophilized. The white powder thus obtained was dissolved in 2 ml of distilled water to make concentrated proactivator B solution. SK of various units was added 0.1 ml each time to this concentrated proactivator B solution, and the mixture was incubated at 37°C for 10 minutes. The lysed area was determined on the standard fibrin plate. As indicated in Fig. 1a, the activity was associated with the units of SK used.

Activator solution was prepared by adding 0.1 ml of 2000 u/ml SK to 0.9 ml of this concentrated proactivator B solution. The mixture was incubated at 37°C for 10 minutes to convert proactivator to activator. This activator solution was

![Fig. 1a](image-url)

**Fig. 1a** Relationship between the fibrinolytic activity and proactivator B adding to various units of SK.

![Fig. 1b](image-url)

**Fig. 1b** Relationship between the fibrinolytic activity and various concentration of activator B activated from proactivator B by SK.
diluted many fold with saline and the activity of each was determined by the lysed area on the standard fibrin plate.

Also in this experiment, a direct relationship between lysed area and the content of the activator was recognized.

F) Inhibitory effects of EACA and t-AMCHA against activity of the activator B.

To determine the inhibition by EACA and t-AMCHA, lysed area caused by activator B was measured on the fibrin plate containing inhibitors. EACA or t-AMCHA was added to fibrinogen in final concentration as shown in Fig. 2 in advance to the addition of thrombin. Fig. 2 indicates that activator activity was inhibited by EACA and t-AMCHA with low concentration, which is the same with the case of activator A.

DISCUSSION

Since the authors had presented two kinds of plasminogen proactivator, namely proactivator A and proactivator B, in 1964, the attention was paid to their significance by some workers, and the variation in infant and in various diseases has been recently reported.

In the study on rabbit’s fibrinolytic components, the authors found that the proactivator corresponding human proactivator A or proactivator B did not exist in rabbit’s plasma, however, that a proactivator-like component was contained.
of which activity was not demonstrated until a very small amount of human euglobulin was added with SK. This component was detected by the gel filtration method in the eluant corresponding human proactivator A fraction. It was presumable that this proactivator-like component was a primitive form of human proactivator A and that proactivator B was unique to human plasma.

There was little doubt as to the existence of proactivator A, since activator A activated with SK had already been separated by gel filtration. Proactivator B, on the other hand, was difficult to separate from plasminogen, accordingly a question arises that fibrinolytic activity of proactivator B fraction demonstrated by adding SK might be caused by the direct activation of plasminogen which did not necessitate proactivator-activator conversion.

In the present paper, an attempt was made first to abolish plasminogen activity in proactivator B fraction in order to solve this difficult question.

Plasminogen contained in proactivator B fraction was resistant to heating in acid reaction but lost activity when heated at 80°C for 20 minutes in neutral or weak alkaline. In spite of such treatment, proactivator B was still preserved. It is assumed that during this process activity of proactivator B was considerably reduced, and yet by utilizing the slight difference between plasminogen and proactivator B in resistance to heat, proactivator B solution freed of plasminogen was presented.

In the experiment using UK, the authors encountered a situation which would possibly lead to the assumption of dual action of UK in fibrinolysis, such as activation on plasminogen and proactivator by the following result. That is, when measured by a standard fibrin plate, the lysis demonstrated with the mixture of UK and proactivator B freed of plasminogen was always bigger than that demonstrated by the same units of UK only (Group A and C in table 5). As to this point, further detailed experiments have been under way.

In the experiment in the system where fibrin, plasminogen and proactivator were sufficient and constant, fibrinolytic activity was determined by the amount of SK and also by the content of the activator. The sum of proactivator B activities presented by the gel filtration pattern of fresh blood was, although containing plasminogen, always bigger than that of proactivator A activities. That is to indicate that proactivator B and activator B are important components in fibrinolytic system in a living body. Plasminogen activating effect of activator B was inhibited by low concentrated EACA or t-AMCHA, as was in the case of activator A.

To seek the factor in a living body which activates those proactivators in place of SK is a next important task. As to this point, the results will be reported elsewhere.
PLASMINOGEN-FREE PROACTIVATOR IN HUMAN PLASMA

CONCLUSION

1) An attempt has been made to obtain proactivator B freed of plasminogen from proactivator B fraction which was isolated from human plasma by Sephadex gel filtration method.

2) Plasminogen contained in proactivator B fraction was labile at heating at pH 4.0-8.0 while proactivator was relatively stable. Accordingly, proactivator B solution freed of prasminogen was obtained from proactivator B fraction by heating at pH 7.0 or 8.0 at 80°C for 20 minutes.

3) Activator B was converted from proactivator B by the activation by SK. The activity observed was associated with the units of SK, and plasminogen converting effect of the activator B was associated with the content of the activator.

4) The activity of activator B was inhibited by EACA and t-AMCHA as similar to the case of activator A.

5) Activation mechanism of UK in plasmin system was discussed.

6) The conclusion is that proactivator B exists in human plasma as well as proactivator A.

REFERENCES
