Effects of gastrointestinal Kampo-hozai and herbs on the *in vitro* activating reaction of the murine pancreatic zymogens

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Abstract

We investigated the effects of some kinds of Kampo-hozai and its component herbs widely used as gastrointestinal drugs on the *in vitro* activation of the murine pancreatic zymogens by the murine intestinal extract. Most of them showed accelerative effects on the activating reaction induced by the intestinal extract, however only Phellodendri Cortex showed a decelerative effect on the activating reaction. Berberine (major component of Phellodendri Cortex) slightly decelerated the trypsin-induced activating reaction, but showed an accelerative effect on the activating reaction induced by non-tryptic intestinal activating factors. However the methanol insoluble fraction showed a remarkably decelerative effect on the activating reaction induced by both trypsin and intestinal activating factors owing to a trypsin inhibitor like property of the fraction.

Key words digestive protease, pancreatic zymogen, drug acting on gastrointestinal disease, Kampo-hozai, Phellodendri Cortex, berberine, inhibitor of trypsin.

Abbreviation Sho-saiko-to (Xiao-Chai-Hu-Tang), 小柴胡湯.

Introduction

Digestive proteases are synthesized and stored as inactive proenzyme forms (zymogens) in the pancreas. After secretion into the duodenum, they are converted into active proteases. In bovine animals, the immediate precursor of trypsin, trypsinogen, is activated after selective cleavage by enterokinase. The trypsin thus formed continues the activation of trypsinogen and induces the activation of the zymogens. In the case of the mouse, it has been reported that several activating factors are present in the intestinal extract.[1)]

On the other hand, many kinds of Kampo-hozai (traditional blended Chinese medicines), aqueous extracts of a mixture of natural crude drugs, and respective aqueous extracts of many kinds of crude drugs have been used as drug therapy for some thousands of years in China, and administered orally as decoction. Some of them have been used to treat patients suffering from gastrointestinal troubles. Since they have been orally administered, investigation of their pharmacological effects on gastrointestinal organs, including the activation of pancreatic zymogens, is of significance.

In this study, we investigated the effects of three kinds of Kampo-hozai, and their constituent herbs which were used as gastrointestinal drugs, on activating reactions of pancreatic zymogens, with the following enzyme assay that pancreatic zymogens and intestinal zymogen-activating factors were mixed with one of such oriental medicines *in vitro*, and the proteolytic activity of the resulting active proteases were measured by the casein-Folin method.[2)] In addition, Phellodendri

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Cortex was studied in detail because of its interesting property.

Materials and Methods

Materials : Casein was obtained from Merck (Rahway, New Jersey, U.S.A.). Trypsin was obtained from Difco Laboratories (Detroit, Michigan, U.S.A.). Other reagents used were analytical grade products obtained from Wako Pure Chemical Industries (Tokyo, Japan).

All Kampo-hozai we used were purchased from Kokando. Amomi Semen, *Amomum xanthioides* WALLICH (縮砂, Thailand), Cinnamomi Cortex, *Cinnamomum cassia* BLUME (桂皮, China), Coptidis Rhizoma, *Coptis japonica* MAKINO (黄連, Fukui Prefecture), Foeniculi Fructus, *Foeniculum vulgare* MILLER (茴香, China), Gentianae Radix, *Gentiana lutea* LINNE (ゲンチアナ, France), Phellodendri Cortex, *Phellodendron amurense* RUPRECHT (黄柏, Niigata Prefecture), Rhei Rhizoma, *Rheum palmatum* LINNE (大黄, China), Scuttellariae Radix, *Scutellaria baicalensis* GEORGI (黄芩, China), Sennae Folium, *Cassia angustifolia* VAHL (センナ, India), Swertiae Herba, *Swertia japonica* MAKINO (センブリ, Nagano Prefecture) and Zingiberis Rhizoma, *Zingiber officinale* ROSCOE (生姜, China) were obtained from Matsubara Funmatsu Yakuhin (Toyama, Japan).

Animal and tissue preparation : Male mice (8 weeks old) of C3H / He strain were starved for 16－20 hours before tissue preparation. Pancreas and intestine from each mice were homogenized separately in 4 volumes of deionized water, at 4℃ using a glass homogenizer. The resulting homogenates were centrifuged at 12,000 × g for 30 minutes to remove small pieces of connective tissue. Supernatants were pooled and used for the enzyme assay.

Preparation of the water extract of the Kampo-hozai and herbs : We used three Kampo-hozai variants in this study (Table I). Each Kampo-hozai (1 packet) was broken into small pieces in a mortar, to this was added 20 ml of deionized water, then mixed and filtered through a sheet of filter paper. Another 20 ml of water was added

Table I Construction of Kampo-hozai.

Kampo-hozai	Component	Weight (mg/3 packets)
Akadama	Geranii Herba (ゲンノショウコ)	530
	Coptidis Rhizoma (黄連)	100
	Phellodendri Cortex (黄柏)	720
	Myricae Cortex (ヨウバイヒ)	200
	Methylenethymol tannin	400
	Extractum Scopoliae (ロートエキス)	50
	Cow Gall (牛胆)	50
Yutan'en	Coptidis Rhizoma (黄連)	70
	Phellodendri Cortex (黄柏)	380
	Swertiae Herba (センブリ)	70
	Gentianae Radix (ゲンチアナ)	200
	Rhei Rhizoma (大黄)	200
	Curcumae Rhizoma (ウコン)	350
	Aloe (アロエ)	
	Cow Gall (牛胆)	
	Malloti Cortex (赤日柏)	2500
Sho-saiko-to	Zingiberis Rhizoma (生姜)	700
	Scutellariae Radix (黄芩)	2100
	Ginseng Radix (人参)	2100
	Zizyphi Fructus (大棗)	2100
	Bupleuri Radix (柴胡)	4200
	Pinelliae Tuber (半夏)	3500
	Glycyrrhizae Radix (甘草)	1400

and the filtration was repeated. The two resulting filtrates were combined and evaporated to 2.0 ml *in vacuo* and pooled. This was used in a 500-fold dilution in the following experiment. Each herb was finely powdered and extracted with 10 volumes (W/W) of deionized water at 100 ℃ for 2 hours. The aqueous extract was filtered through a sheet of filter paper and the filtrate was 5-fold diluted for the experiment (only in the case of Phellodendri Cortex and Coptidis Rhizoma, it was 10-fold diluted).

Preparation of the methanol insoluble fraction of Phellodendri Cortex[3] : Phellodendri Cortex was extracted with methanol and filtrated with filter paper, and the resulting filtrate was discarded. This extraction was repeated several times in order to remove methanol soluble components such as berberine. The resultant was dried and extracted repeatedly with 50% acetonitrile. Filtrates were combined and evaporated. The water soluble fraction of the evaporated extract was collected (the yield was approximately 2.0%).

Activation of pancreatic digestive protease zymogens in vitro : We used the murine pancreatic and intestinal extracts as sources of pancreatic zymogens and their activator, respectively. One-half milliliter of various concentrations of the intestinal extract, 0.5 ml of 1.0% (W/V) pancreatic extract, and 1.0 ml of the extract of the Kampo-hozai or the constituent herb were mixed, and then incubated at 37℃ for 60 min to activate the zymogens.

Enzyme assay : To estimate the activity of activated digestive proteases or trypsin, we used the modified Anson's method[4] (casein - Folin method).[2] One milliliter of substrate solution (1.0% (W/V) casein) was added to activated proteases or trypsin, and incubated at 37℃ for 30 minutes. The reaction was inactivated by addition of 1.0 ml of 0.8 M trichloroacetic acid (TCA), and the mixture was centrifuged at 1,700×g for 10 minutes. One millilier of supernatant, 5.0 ml of 0.4 M sodium carbonate and 1.0 ml of 1.0 N phenol reagent were mixed, and after 20 minutes the optical density was read with a spectrophotometer at 660 nm. One unit of protease activity was defined as the amount of split tyrosine residue liberted per minute under standard conditions. One unit corresponded to the same intensity of the color obtained from 1 milliequivalent of tyrosine treated by the phenol reagent.

Results

Effects of Kampo-hozai

Figure 1 shows the effects of three kinds of Kampo-hozai (Table I) used as gastrointestinal drugs, on activating reactions of pancreatic zymogens. All these drugs accelerated reactions.

Effects of herbs

The initially tested herbs were those used as bitter stomachic ; Coptidis Rhizoma (Fig. 2), Gentianae Radix and Swertiae Herba (Fig. 3) accelerated reactions, while Phellodendri Cortex had

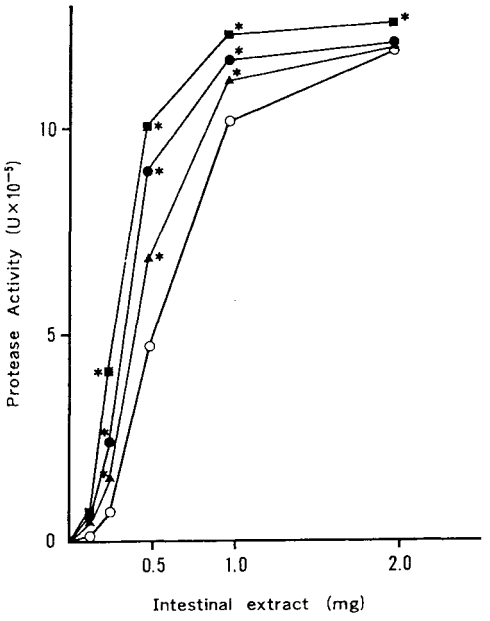

Fig. 1 Effects of Akadama, Yutan'en and Sho-saiko-to on an *in vitro* activating reaction of pancreatic zymogens induced by intestinal zymogen activating factors.
○ : control, ● : Akadama, ▲ : Yutan'en, ■ : Sho-saiko-to. Each point represents the mean of three experiments. Each standard error was smaller than the range covered by the symbols. Significantly different from control at *) $p < 0.01$.

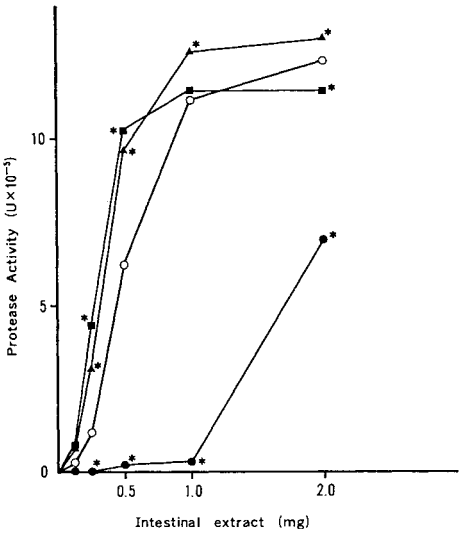

Fig. 2 Effects of Phellodendri Cortex, Coptidis Rhizoma and berberine sulfate on an *in vitro* activating reaction of pancreatic zymogens induced by intestinal zymogen activating factors.
○ : control, ● : Phellodendri Cortex, ▲ : Coptidis Rhizoma, ■ : berberine sulfate. Each point represents the mean of three experiments. Each standard error was smaller than the range covered by the symbols. Significantly different from control at *) $p<0.01$.

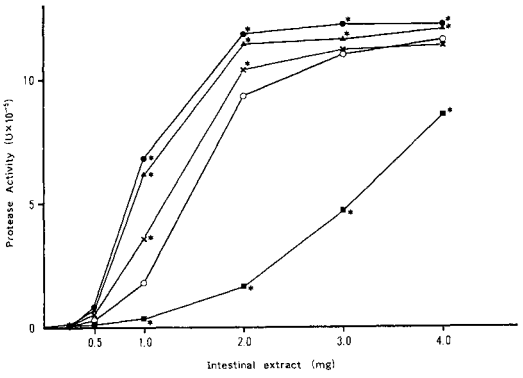

Fig. 3 Effects of Gentianae Radix, Swertiae Herba, Rhei Rhizoma and Sennae Folium on an *in vitro* activating reaction of pancreatic zymogens induced by intestinal zymogen activating factors.
○ : control, ● : Gentianae Radix, ▲ : Swertiae Herba, ■ : Rhei Rhizoma, X : Sennae Folium. Each point represents the mean of three experiments. Each standard error was smaller than the range covered by the symbols. Significantly different from control at *) $p<0.01$.

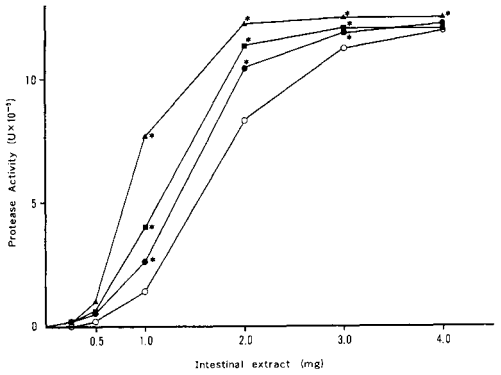

Fig. 4 Effects of Cinnamomi Cortex, Foeniculi Fructus and Zingiberis Rhizoma on an *in vitro* activating reaction of pancreatic zymogens induced by intestinal zymogen activating factors.
○ : control, ● : Cinnamomi Cortex, ▲ : Foeniculi Fructus, ■ : Zingiberis Rhizoma. Each point represents the mean of three experiments. Each standard error was smaller than the range covered by the symbols. Significantly different from control at *) $p<0.01$.

a decelerating effect (Fig. 2). We then tested herbs used as aromatic stomachic. Cinnamomi Cortex, Foeniculi Fructus and Zingiberis Rhizoma accelerated reactions (Fig. 4). No effect was observed with Amomi Semen as a stomachic and Scutellariae Radix as an anti-inflammatory drug. Althought both Sennae Folium and Rhei Rhizoma were used as a laxative, the former showed an accelerative effect, while the latter showed a decelerative effect, as did Phellodendri Cortex (Fig. 3).

Effects of berberine sulfate and methanol insoluble fraction of Phellodendri Cortex

Since Phellodendri Cortex and Coptidis Rhizoma, in spite of the fact that each of them contains berberine as a major component, showed the countereffect on the activating reactions as mentioned above. We tested the effects of berberine sulfate and the methanol insoluble fraction of Phellodendri Cortex. We obtained the results that berberine sulfate showed an accelerative effect (Fig. 2), while the methanol insoluble fraction showed a remarkably decelerative effect as did the water extract of Phellodendri Cortex (Fig. 5).

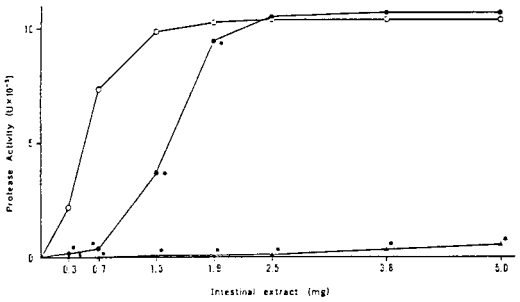

Fig. 5 Effect of the methanol insoluble fraction of Phellodendri Cortex on an *in vitro* activating reaction of pancreatic zymogens induced by intestinal zymogen activating factors.
○ : control, ● : methanol insoluble fraction of Phellodendri Cortex (0.5 mg), ▲ : (2.5 mg). Each point represents the mean of three experiments. Each standard error was smaller than the range covered by the symbols. Significantly different from control at *) $p<0.01$.

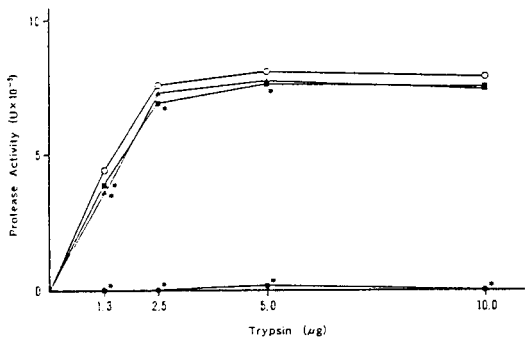

Fig. 6 Effects of Phellodendri Cortex, Coptidis Rhizoma and berberine sulfate on a trypsin-induced *in vitro* activating reaction of pancreatic zymogens.
○ : control, ● : Phellodendri Cortex, ▲ : Coptidis Rhizoma, ■ : berberine sulfate. Each point represents the mean of three experiments. Each standard error was smaller than the range covered by the symbols. Significantly different from control at *) $p<0.01$.

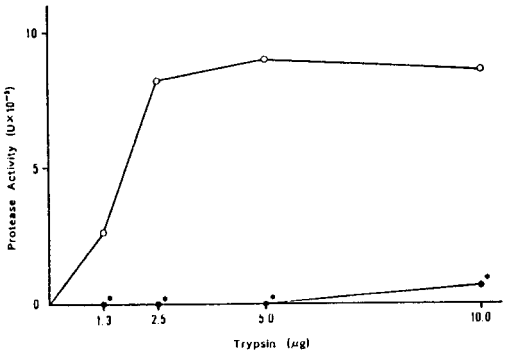

Fig. 7 Effect of the methanol insoluble fraction of Phellodendri Cortex on a trypsin-induced *in vitro* activating reaction of pancreatic zymogens.
○ : control, ● : methanol insoluble fraction of Phellodendri Cortex (0.5 mg). Each point represents the mean of three experiments. Each standard error was smaller than the range covered by the symbols. Significantly different from control at *) $p<0.01$.

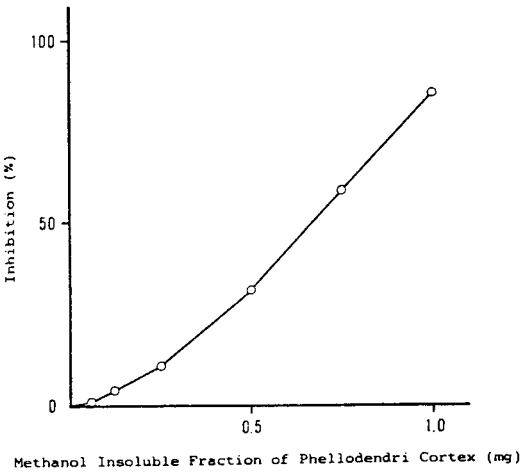

Fig. 8 Inhibitory effect of the methanol insoluble fraction of Phellodendri Cortex on the proteolytic activity of trypsin (20.0 μg).
Each point represents the mean of two experiments.

Effects of Phellodendri Cortex, Coptidis Rhizoma, berberine sulfate and the methanol insoluble fraction of Phellodendri Cortex on trypsin-induced activating reactions

The trypsin-induced reaction was remarkably decelerated by both Phellodendri Cortex (Fig. 6) and its methanol insoluble fraction (Fig. 7). However, Coptidis Rhizoma and berberine sulfate showed a slightly decelerative effect on the trypsin-induced reaction (Fig. 6), in spite of the fact that they showed an accelerative effect on the reaction induced by intestinal activating fac-

tors.

Effect of methanol insoluble fraction Phellodendri Cortex on the proteolytic activity of trypsin

The above-mentioned results suggested an inhibitory effect of the methanol insoluble fraction of Phellodendri Cortex on limited proteolysis ; next, we investigated the effect on the non-limited proteolysis of trypsin using casein as substrate. The obtained results indicated that the fraction showed strong dose-dependent inhibition on the proteolytic activity of trypsin (Fig. 8).

In Fig. 9, the kinetics of inhibition of proteolysis were plotted according to Lineweaver and Burk. Proteolytic activity of trypsin was inhibited by the fraction in an uncompetitive fashion, both K_m and V_{max} values being reduced in the presence of the fraction.

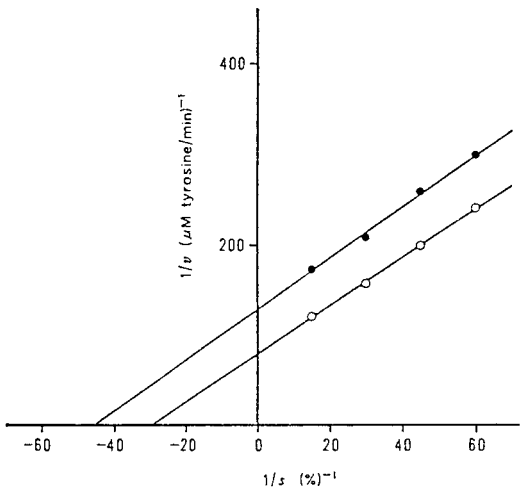

Fig. 9 Lineweaver-Burk plots of proteolytic activity of trypsin in the presence of the methanol insoluble fraction of Phellodendri Cortex.
○ : control, ● : methanol insoluble fraction of Phellodendri Cortex. Each point represents the mean of two experiments.

Discussion

In the present study, we investigated the effects of certain kinds of Kampo-hozai and its component herbs on the activating reactions of murine pancreatic zymogens with an *in vitro* activation method. According to the results, all Kampo-hozai and herbs used as stomachic which we had tested, except Phellodendri Cortex and Amomi Semen, accelerated reactions induced by zymogen activating factors localized in the intestinal mucosa. We paid attention to Phellodendri Cortex because of following two reasons. In spite of the fact that it was abundantly included in gastrointestinal Kampo-hozai, the effect of Phellodendri Cortex was opposite those of Kampo-hozai, and in spite of the fact that Phellodendri Cortex and Coptidis Rhizoma contained berberine as a major component and are used similarly today in Japan, their effects on activating reactions were opposite. Therefore, we investigated the effects of berberine and the methanol insoluble fraction of Phellodendri Cortex on the activating reactions. Berberine showed an accelerative effect, however the methanol insoluble fraction showed a remarkably decelerative effect as did the water extract of Phellodendri Cortex on the activating reaction induced by intestinal activating factors.

On the other hand, since trypsin is one of the most famous activators of pancreatic zymogens, we investigated the effects of Phellodendri Cortex, beberine sulfate, the methanol insoluble fraction and Coptidis Rhizoma on trypsin-induced activating reactions of pancreatic zymogens. Both Phellodendri Cortex and its methanol insoluble fraction remarkably decelerated reactions as well as the reaction induced by intestinal activating factors. However both berberine sulfate and Coptidis Rhizoma showed slightly decelerative effects. In addition, we showed the methanol insoluble fraction had a uncompetitive trypsin inhibitory property. These results suggested that the accelerative effect of berberine occurred in the activating reaction induced by non-tryptic zymogen activating factors which was localized in the intestinal mucosa, and the decelerative effect of the methanol insoluble fraction owing to its trypsin inhibitory property suppressed the accelerative effect, and resulted in the decelerative effect of the water extract of Phellodendri Cortex.

As mentioned above, *in vitro* conversion of

the inactive type of digestive protease to the active type, which acts in the intestinal organ, was affected by some kinds of gastrointestinal oriental medicines. Kampo-hozai, which showed accelerative effects, are composed of not only accelerative components (herbs *e.g.* Coptidis Rhizoma), but are also abundant in strongly decelerative components (*e.g.* Phellodendri Cortex). Similarly, Phellodendri Cortex is composed of not only decelerative components, but also accelerative components (*e.g.* berberine being a major component). We think such a factor brings on homeostasis, which is a characteristic feature of oriental medicines. In oriental medicines, drugs neutralize the unbalanced condition of a patient's full body, not only a part of the disease, and we think such drugs need to be homeostatic within themselves to act properly as oriental medicines.

Since Phellodendri Cortex is frequently used as a source of berberine today in Japan, as being a major component, and is not so distinguishably used from Coptidis Rhizoma, they have not been noticed very much except for the major component. We thought it was necessary to investigate in more detail not only the major component, but also non-major components to throw light on the homeostatic balance of oriental medicines. Therefore, we have started to try to purify this inhibitor of trypsin.

Numerous components in Kampo-hozai and the constituent herbs with various interactions make it quite difficult to elucidate its mechanism of action. However, the pharmacological actions should be elucidated since hundreds of Kampo-hozai are widely applied in Japan and China. This type of study is useful as a preliminary one to throw scientific light on the theory called "Sho."

和文抄録

健胃整腸薬として用いられる3種の方剤及び単独でも同様に用いられるそれらの構成生薬について，マウス膵臓由来プロテアーゼザイモーゲンのマウス小腸抽出液による活性化反応に及ぼす影響を検討した。その結果，それらの多くは促進効果を示したが，黄柏のみが抑制効果を示した。更に，黄柏の主成分であるベルベリンは，トリプシンによるザイモーゲンの活性化反応に対し弱い抑制効果を示したが，トリプシン以外の小腸由来ザイモーゲン活性化因子による活性化反応に対し，促進効果を示した。しかし，黄柏のメタノール不溶性分画はトリプシンに対し阻害作用を示し，トリプシン及び小腸由来ザイモーゲン活性化因子による活性化反応に対し，強い抑制効果を示した。

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