bonds/g); [I], insoluble substrate concentration (mg/mL); [IE], insoluble substrate concentration, resistant to hydrolysis (mg/mL); k_I, reaction rate constant for insoluble pool I (mL/mg per min); k_S, reaction rate constant for soluble pool S (mL/mg per min); K_{mi}, Michaelis–Menten constant for pool i (mg/mL); [P], concentration of soluble low molecular weight peptides, not precipitated by TCA (mg/mL); r_I, rate of hydrolysis of substrate I (mg/mL per min); r_S, rate of hydrolysis of substrate S (mg/mL per min); [S], soluble protein/high molecular weight peptides, precipitated by TCA (mg/mL); t, time (min).

Registry No. Subtilisin, 9014-01-1.

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Monoclonal Antibody-Based Enzyme Immunoassay of the Bowman–Birk Protease Inhibitor of Soybeans

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Monoclonal antibodies that bind the Bowman–Birk protease inhibitor (BBI) of soybeans were derived from mice inoculated with an emulsion of native, un-cross-linked protein. The antibodies did not cross-react with the soybean Kunitz trypsin inhibitor or the homologous inhibitors from lima beans and chickpeas. One epitope of BBI, which is destroyed by heat and by disulfide-modifying reagents, was defined. Monoclonal antibodies that recognize this epitope can be used for specific recognition of native BBI in the presence of denatured forms, both in purified samples and in extracts of soybean cultivars and processed foods. An enzyme-linked immunosorbent assay (ELISA) for BBI was developed with these antibodies, and the results from the ELISA agreed with enzymatic assays of inhibitory activity in heat-treated soy meal. The monoclonal antibodies were also effective for affinity isolation of BBI from soy isolates. The demonstration that BBI retained its native conformation following conjugation with enzyme and that the antibody was stable during immunoaffinity procedures indicates that these immunochemical methods could readily be used for screening plant germplasm and in biomedical studies.

The protein of soybeans (*Glycine max*) is widely used in human foods in a variety of forms including infant formulas, soy protein isolates, soy flour, textured soy fibers, soy sauce, and tofu as well as in animal feeds. Protease inhibitors constitute about 6% of the proteins of soybeans (Rackis et al., 1986). The two major inhibitors are the Kunitz trypsin inhibitor (KTI) and the Bowman–Birk inhibitor (BBI), a low molecular weight (M_r 8000) double-headed inhibitor of chymotrypsin and trypsin (Birk, 1985). The presence of inhibitors of digestive enzymes in soybeans impairs their nutritional quality and promotes the development of pancreatic nodular hyperplasia and acinar cell adenomas in rats (Gumbmann et al., 1986). On the other hand, recent evidence suggests that BBI may

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function as an anticarcinogen through its interaction with a cellular serine protease (Yavelow et al., 1983; Billings et al., 1987, 1988).

Soybean protein products, properly processed, serve as an excellent source of low-cost, high-quality protein for human needs (Rackis and Gumbmann, 1981). The sulfur-rich protease inhibitors contribute substantially to the potential nutritional value of soy-derived protein and may also serve to reduce crop predation by insects (Green and Ryan, 1972). Soybean protease inhibitors are inactivated during commercial processing by heat treatments, which leave 10–15% residual inhibitory activity. This activity is difficult to characterize because enzymatic assays give inaccurate results and do not differentiate among the various specific and nonspecific inhibitors present (Lehnhardt and Dills, 1984). Recently, a genetic approach has been used to remove the Kunitz trypsin inhibitor (KTI) from soybeans (Hymowitz, 1986). With either approach, it is necessary to measure low levels of protease inhibitors in foods. As the evidence for both the toxicity and beneficial effects accumulates, better methods are needed to measure specific protease inhibitors.

Although antibodies to BBI have been reported (Offir et al., 1971; Hwang et al., 1977; Tan-Wilson et al., 1982; Horisberger and Tacchini-Vonlanthen, 1983), the native molecule was thought to be insufficiently antigenic to elicit useable antisera. To increase the antigenicity, BBI was cross-linked with glutaraldehyde and used by these workers to elicit antibodies in rabbits. However, these polyclonal antibodies were low in affinity, and the resulting immunoassays (Hwang et al., 1977) were not sensitive enough for complex samples such as processed foods. In contrast to these previous findings, we found that un-cross-linked BBI is sufficiently antigenic in mice and can be used to generate high-affinity monoclonal antibodies. This report describes immunoassay methods that complement our previously described ELISA for KTI (Brandon et al., 1988) to enable characterization of the major protease inhibitory activities in soybean cultivars and processed foods. In addition, we found these monoclonal antibodies effective for immunoaffinity purification of BBI.

MATERIALS AND METHODS

Protease Inhibitors. BBI (Birk, 1985) and chickpea inhibitor (Smirnoff et al., 1976) were provided by Prof. Y. Birk (Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot, Israel). KTI and lima bean inhibitor (LBI) were obtained from Sigma Chemical Co. (St. Louis, MO). Standard solutions were prepared in phosphate-buffered saline (PBS, 150 mM NaCl, 5 mM sodium phosphate, pH 7.0) or PBS with 0.01% NaN_3 (PBS–N_3). The solutions were adjusted to 1 mg/mL based on A_{280} [1 mg/mL = 1.03 for KTI and 0.44 for BBI and LBI (Kassell, 1970)] and were stored as aliquots at −20 °C. Treatment of inhibitors at elevated temperatures and with disulfide-modifying reagents was described, and trypsin inhibitor units were defined previously (Friedman et al., 1982; Friedman and Gumbmann, 1986). In brief, a sample consisting of 10 mL of 1 mg/mL BBI in 0.5 M Tris-Cl buffer, pH 8.5, was treated for 1, 2, or 3 h without or with 1 mg/mL *N*-acetylcysteine (NAC) or 1 mg/mL sodium sulfite. BBI was cross-linked with glutaraldehyde (Reichlin et al., 1970) and then reduced with sodium borohydride. The cross-linking was verified by polyacrylamide gel electrophoresis in the presence of dodecyl sulfate.

Enzymatic Assays. Inhibition of trypsin activity was measured at room temperature in 0.4 M Tris-Cl, 0.01 mM $CaCl_2$, pH 8.1, and with 1 mM (*p*-tolylsulfonyl)-L-arginine methyl ester as substrate (Hummel, 1959). Inhibition of chymotrypsin activity was determined at room temperature in 0.04 M Tris-Cl, 0.05 M $CaCl_2$, pH 7.8, with 0.5 mM benzoyl-L-tyrosine ethyl ester as substrate (Hummel, 1959).

Soy Meal Extracts. A commercially grown cultivar (Williams-82) was compared to a low trypsin inhibitor isoline L81-4590 (Hymowitz, 1986) provided by Prof. T. Hymowitz (University of Illinois, Urbana, IL). The absence of KTI in this isoline, determined originally by gel electrophoresis, was confirmed by enzymatic assay and ELISA (manuscript in preparation). Soybeans were ground in a Udy mill, sieved through No. 60 mesh, and subjected to heat treatment in an autoclave at 121 °C. Tris-Cl buffer (0.5 M, pH 8.5) was added (10 mL/300 mg of soy meal), and the slurry was homogenized with a Tissumizer (Tekmar Co., Cincinnati, OH). The homogenate was then stirred for 2 h at room temperature and clarified by centrifugation at 4000*g* for 10 min. Protein isolates were prepared from raw soy meal by extraction, acid fractionation, ammonium sulfate precipitation, and gel filtration on Sephadex G-100 (Pharmacia, Inc., Piscataway, NJ) as described by Hwang et al. (1977), but without the addition of mercaptoethanol to the buffers.

Immunochemistry. *Antibodies.* Antisera were prepared from blood drawn 1 week after two intraperitoneal inoculations of BALB/c mice with 50 μg of either native BBI or glutaraldehyde-treated BBI emulsified with complete Freund's adjuvant. Monoclonal antibodies were derived from those mice inoculated with native BBI (Oi and Herzenberg, 1980) and the myeloma cell line P3-X65-Ag8.653 (Kearney et al., 1979). Antibodies were purified by ammonium sulfate fractionation and ion-exchange chromatography from ascitic fluid obtained from BALB/c mice previously inoculated intraperitoneally with the appropriate hybridoma cell line. Isotype was determined using reagents from Southern Biotechnology (Birmingham, AL).

Labeled BBI. BBI was conjugated to horseradish peroxidase (HRP) according to Nakane and Kawaoi (1974), with slight modification (Brandon et al., 1988), using equimolar quantities of the proteins. Unconjugated BBI was removed by gel filtration using Sephadex G-100. The resulting conjugate (1.6 mg/mL) was stored refrigerated in PBS containing 10 mg/mL bovine serum albumin (BSA) and 0.1% merthiolate and was used at a dilution of 1:5000.

Immunoaffinity Methods. One gram of cyanogen bromide activated agarose (Sepharose 4B, Pharmacia) was washed and swollen in 1 mM HCl. Monoclonal antibody 238 (19 mg in 6 mL of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) was added to the agarose and incubated for 2 h at room temperature with shaking. The agarose was then washed; unreacted sites were blocked by treatment with 1 M ethanolamine, pH 8.0, for 4 h; and the agarose was washed again and stored in PBS–N_3. The binding capacity of the antibody–agarose matrix was determined to be 10 μg of BBI/mL of matrix. A 1-mL of sample of soy isolate was applied to a column containing 3.5 mL of the agarose matrix and allowed to bind for 20 min. The column was then washed with PBS, and the flow-through was collected. Retained proteins were then eluted with 0.1 M acetic acid. Column fractions were dialyzed against water, lyophilized, redissolved in 0.1–0.2 mL of PBS, and analyzed by electrophoresis in a polyacrylamide gel (12% acrylamide, 0.3% methylene bisacrylamide, 0.4 M Tris-Cl, pH 8.8, containing 4 M urea). Amido black was used to stain the proteins.

Immunoassays. *Antibody Titrations and Inhibition ELISA.* These assays were performed with use of polystyrene assay plates (Nunc, Roskilde, Denmark), as described previously (Brandon et al., 1987, 1988). BBI was coated on the assay wells by incubating 100 μL/well of protein at 5 μg/mL for 4 h at room temperature or overnight at 4 °C. The plates were washed, blocked by incubation with 200 μL/well of PBS containing 0.05% Tween-20 and 10 mg/mL BSA (BSA–PBS–Tween), and used immediately or stored for up to 2 weeks at 4 °C with wells filled with PBS–N_3. All ELISA procedures were conducted at room temperature. For inhibition ELISA, antibodies were preincubated for 1 h in BSA–PBS–Tween with appropriate concentrations of samples to be analyzed. After application of the incubated solutions to the wells (100 μL/well for 1 h), bound IgG was detected with HRP-labeled rabbit anti-mouse IgG (Zymed Laboratories, South San Francisco, CA). HRP activity was measured in 60 mM sodium citrate buffer, pH 4.2, containing 6.7 mM H_2O_2 and 1 mM 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid). The absorbance was determined at 415 nm on a plate reader (Model 308, Bio-Tek Instruments, Winooski, VT) after an appropriate interval (usually 15 min) or after the reaction was terminated by the addition of 10% sodium dodecyl sulfate.

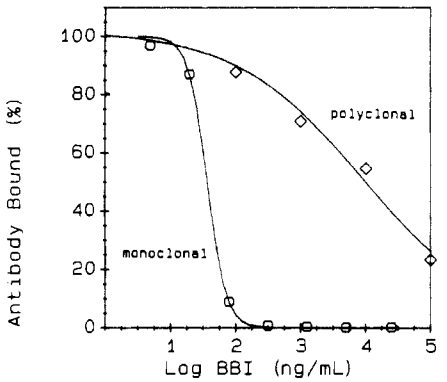

Figure 1. Inhibition ELISA of BBI with polyclonal antibodies or monoclonal antibody 238. The assays were conducted with antiserum at 1:500 dilution or antibody 238 at 0.4 μg/mL.

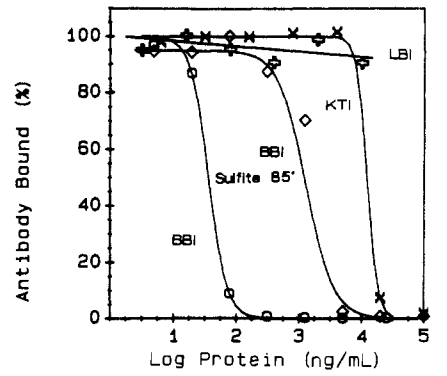

Figure 2. Inhibition ELISA to determine specificity of antibody 238. The reactivity of antibody with BBI was compared with its binding to LBI, KTI, and BBI previously treated with sodium sulfite at 85 °C for 2 h.

Competition ELISA. The assay was conducted on poly(vinyl chloride) assay plates (Costar, Cambridge, MA) coated with purified IgG anti-BBI (10 μg/mL, 50 μL/well). Unknowns or standard samples containing BBI were premixed with equal volumes of BBI–HRP and incubated in the assay wells (50 μL/well, 1–2 h at room temperature with shaking). The binding reaction was complete within 1 h, with no change in binding between 1 and 2 h. Unbound HRP conjugate was removed by washing and rinsing the plates, and the bound HRP was visualized as described above.

RESULTS AND DISCUSSION

Antisera to BBI. All mice inoculated with either native or glutaraldehyde-treated BBI produced anti-BBI antibodies. In contrast to previous reports, there was no indication that glutaraldehyde-treated BBI elicited a better response than native BBI, as measured by ELISA using assay wells coated with the native form. The specificity of the antisera was confirmed by inhibition ELISA. A typical assay curve obtained with these polyclonal antibodies is presented in Figure 1. All subsequent studies were performed with use of antibodies derived from mice inoculated with native BBI.

Monoclonal Antibodies to BBI. Antibody 238 was selected for further characterization and determined to be IgG_1. The apparent high affinity of this monoclonal antibody resulted in an ELISA sensitivity 100-fold greater than could be obtained with polyclonal antibodies (Figure 1). The affinity was reduced by 97% for BBI denatured by treatment with sodium sulfite at 85 °C for 2 h (Figure 2). The antibody did not bind to LBI measurably, and its cross-reaction with KTI was about 0.1%. This cross-reactivity with commercial preparations of KTI varied from lot to lot and was reduced following further purification of the KTI by gel filtration. Thus, the binding is due to impurities—presumably BBI—which contaminate samples of KTI. The antibody did not bind to the BBI-like inhibitor from chickpeas (not shown).

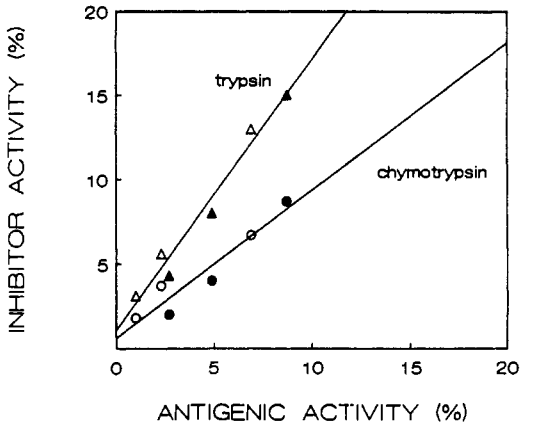

Figure 3. Correlation of BBI antigenic activity determined by ELISA and enzymatic activity determined by inhibition of chymotrypsin ($r = 0.96$) and trypsin ($r = 0.98$). Solid symbols depict NAC-treated samples, and open symbols depict samples treated with sodium sulfite.

Table I. Chymotrypsin and Trypsin Inhibition by Enzyme Assays and BBI Content by ELISA in Two Soybean Cultivars

		% activity remaining		
soybean var.	autoclaving time, min	chymotrypsin inhibn	trypsin inhibn	BBI (ELISA)
Williams-82 (*Ti*[a] *Ti*[a])	10	83	64	84
	20	38	26	1.9
	30	18	17	1.4
L81-4590 (*ti ti*)	10	40	83	59
	20	10	9.3	1.7
	30	7.0	2.3	0.09

[a] The Williams control sample inhibited 150 chymotrypsin units and 4800 trypsin units/g and contained 2.9 mg of BBI/g by ELISA. L81-4590 meal inhibited 143 chymotrypsin units and 2500 trypsin units/g and contained 3.0 mg of BBI/g by ELISA

Comparison of Enzymatic Assay and ELISA of BBI. The relationship among the enzyme inhibitory activities and ELISA was further studied with BBI treated at 85 °C in buffer only, or in the presence of NAC or sodium sulfite. The resulting samples were assayed for trypsin and chymotrypsin inhibition and for activity in the inhibition ELISA, with antibody 238. Relative ELISA activity was calculated as follows. The midpoint of the inhibition curve was used to determine the concentration of sample that inhibits antibody binding by 50% (I_{50}). The I_{50} was also computed for a control sample. Antigenic activity = $[I_{50}(\text{control}) \times 100]/I_{50}(\text{sample})$. Figure 3 shows that there is excellent agreement between the ELISA results and the enzymatic assay. Loss of activities was progressive over the 3-h time course of the experiment. The NAC and sodium sulfite treated samples lost 85–90% of their inhibitory activities within 1 h. Samples treated at 85 °C without a disulfide-modifying reagent (not shown on graph) retained 69–87% of trypsin inhibitory activity, 60–75% of chymotrypsin inhibitory activity, and 100% of antigenic activity. It thus appears that the antibody recognizes the native structure of BBI, which is most affected by disruption of disulfides.

Use of the Monoclonal Antibodies To Measure BBI in Soybean Meal. Two cultivars were analyzed for BBI by ELISA using antibody 238 and for trypsin and chymotrypsin inhibitory activities. Table I shows that the meal from L81-4590 contains about half the initial trypsin inhibitory activity of the Williams sample, consistent with the absence of KTI in this cultivar. Both the chymotrypsin

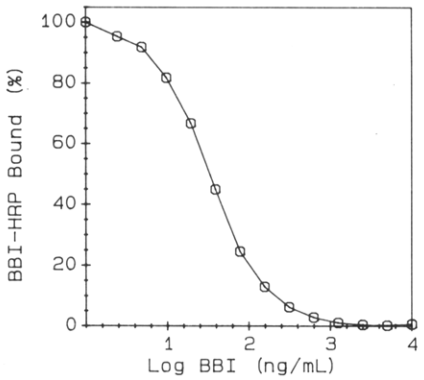

Figure 4. Competitive ELISA of BBI with BBI–HRP conjugate and antibody 238 as solid phase.

and trypsin inhibitory activities appear more heat-labile in L81-4590, in agreement with previous results (Liener and Tomlinson, 1981), with more than 90% inactivation after 20 min. Further, ELISA indicates that BBI is inactivated more rapidly than the total trypsin or chymotrypsin inhibitory activities. The differences between ELISA and enzymatic results are greatest for the samples with very low residual activity and may reflect the contributions of other minor protease inhibitors (Tan-Wilson et al., 1987) and nonspecific inhibition by other soy proteins and components such as phytate and fats.

ELISA Using Labeled BBI. Figure 4 illustrates an alternative immunoassay for BBI with BBI–HRP in competitive ELISA. In this assay, labeled BBI competes with BBI in the samples for a limited number of antibody combining sites on the solid phase. This format is faster than the inhibition ELISA described above, since it combines the sample application and labeling steps. The competitive ELISA had a wider working range than the inhibition assay, making sample preparation less difficult and increasing the precision. A possible explanation for this difference is that enzyme-conjugated BBI molecules are not homogeneous in their interaction with the antibody combining site. An ELISA format using biotinylated BBI was also practical (not shown), confirming that the epitope recognized by antibody 238 does not involve the presumptive lysine residue(s) modified by the labeling reactions.

Immunoaffinity Fraction of Soy Isolate. A soy isolate was fractonated on an immunoaffinity column prepared with antibody 238. Figure 5 illustrates the electrophoretic analysis of two separate experiments. The retained fractions had one component with the mobility and antigenicity of native BBI.

CONCLUSIONS

The results demonstrate that native BBI is immunogenic in mice, elicits an antibody response, and activates sufficient numbers of spleen cells to permit hybridoma formation by conventional methods. Previous workers (Hwang et al., 1977; Tan-Wilson et al., 1982; Horisberger and Tacchini-Vonlanthen, 1983) utilized glutaraldehyde-treated BBI for immunizations and have stated that native BBI is not sufficiently immunogenic to elicit suitable antisera. Use of ELISA may have permitted us to detect antibodies that were overlooked by less sensitive techniques such as immunodiffusion. It is also possible that the BALB/c mouse responds to native BBI better than the rabbit. The efficacy of the ELISA for BBI implies that BBI retained its native antigenicity when bound directly to plastic assay dishes and when conjugated with HRP.

Antibody 238 was shown to be specific for native BBI and not cross-reactive with heat-treated BBI or BBI

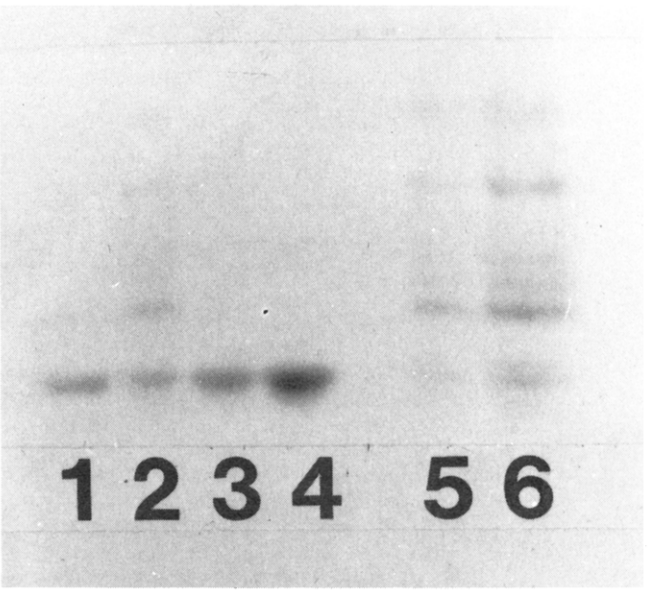

Figure 5. Electrophoretic analysis of soy isolate fractionated by immunoaffinity chromatography on antibody 238 coupled to agarose. Samples: (1) BBI, (2) soy isolate, (3) retained fraction (experiment 1), (4) retained fraction (experiment 2), (5) flow-through (experiment 1), (6) flow-through (experiment 2).

treated with agents that disrupt disulfide bonds. Noteworthy is the lack of cross-reactivity of the antibody with LBI and chickpea inhibitor, all members of the Bowman–Birk family of inhibitors (Laskowski and Kato, 1980). The specificity and high apparent affinity of the antibody enabled the specific detection of low levels of active BBI in heat-denatured samples and in soybean cultivars.

These ELISA methods could be applied in the analysis of foods by industry or regulatory agencies. Active BBI remaining after thermal treatment or after fractionation of soy isolates could be quantitated by ELISA. In addition, the relatively stable BBI molecule could be used as a marker to detect soy protein as an adulterant in meat products.

As a research tool for plant geneticists, the ELISA could be used to detect the product of the BBI gene or closely related genes that appear to be widely distributed in the plant kingdom (Norioka et al., 1988). For example, levels of BBI in soy seeds used in breeding studies could be measured by ELISA. Since it may be desirable to eliminate BBI activity while retaining the high sulfur content of the BBI polypeptide, antibodies to BBI might detect altered forms of BBI with modified reactive sites, but similar three-dimensional structures. Cultivars containing such structures could have higher nutritional value than conventional cultivars and would require less thermal processing. It might also be possible to detect an altered form of BBI that still inhibits chymotrypsin and thus may still be anticarcinogenic, but lacks the pancreatic toxicity associated with trypsin inhibitors.

The antigenicity of BBI, exemplified by its interaction with antibody 238, also offers a possible means of targeting BBI to specific cellular sites. Thus, a bispecific antibody—with one site specific for a cellular antigen and one site identical with that of antibody 238—would provide a well-defined cross-linking agent. Our results indicate that antibody 238 can be used for affinity purification of BBI, suggesting the feasibility of antibody-directed targeting of the inhibitor.

As discussed above, the protease inhibitors of soybeans may have desirable nutritional and pharmacological properties, as well as potential pancreatic toxicity. Use

of monoclonal antibody methods to analyze these inhibitors in processed foods and in new cultivars could help to provide nutritious, healthful soy products for human and animal consumption.

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