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α-GLUTAMYL-β-NAPHTHYLAMIDE HYDROLASE OF RABBIT SMALL INTESTINE

LOCALIZATION IN THE BRUSH BORDER AND SEPARATION FROM OTHER BRUSH BORDER PEPTIDASES

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SUMMARY

About 70 % of the total mucosal enzymatic activity hydrolyzing α-L-glutamyl-β-naphthylamide in the rabbit small intestine is present in the brush border; the specific activity in this subcellular fraction is 7 times higher than that of the homogenate. Similar results are obtained for L-leucyl-β-naphthylamide hydrolase.

The enzyme activity is efficiently solubilized by papain digestion and is clearly separated from L-leucyl-β-naphthylamide hydrolase by chromatography on concanavalin A-Sepharose. It probably represents a digestive peptidase, different from the other known peptide hydrolases of the digestive surface of the small intestine.

INTRODUCTION

The bulk of intestinal peptidase activities appears to be in the sap cell, when di- and tripeptides are used as substrates [1–6]. Nevertheless, the brush border of the small intestine does split some di- and tripeptides with high specific activity, suggesting that this subcellular organelle plays a role in the terminal digestion of proteins [1, 7–9].

β-Naphthylamides of L-amino acids are useful substrates for studying brush border peptidases, as they are mainly hydrolyzed by brush border enzymes. In rat intestine, β-naphthylamides of L-amino acids are hydrolyzed more actively by particle-bound enzyme(s), with the only exception of L-arginyl- and L-lysyl-β-naphthylamide, which are predominantly split by a "soluble" enzyme [10]. L-Leucyl-β-naphthylamide was proved to be an ideal marker substrate for the major brush border peptidase, which is an oligoaminopeptidase [11, 12]. γ-L-Glutamyl-β-naphthylamide is a good substrate for the assay of a different peptidase of the brush border, which is able to split γ-glutamyl bonds in peptides [9].

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Glenner et al. [13] described an enzymatic activity in mammalian tissues (aminopeptidase A) hydrolyzing the N-terminal L-dicarboxylic amino acid residues of both β-naphthylamides and peptides. This enzymatic activity has been recently localized in the brush border of rabbit kidney [14].

In this study, using α-L-glutamyl-β-naphthylamide as substrate, we demonstrate (1) that aminopeptidase A of rabbit small intestine is mainly located in the brush border and (2) that it can be separated from L-leucyl-β-naphthylamide hydrolase. Therefore it probably represents a digestive peptidase different from the other known peptide hydrolases of the digestive surface of the small intestine.

EXPERIMENTAL

Rabbit intestinal brush border

Intestinal mucosa was obtained from adult male rabbits of the New Zealand strain.

To study the subcellular distribution of the enzymatic activities, brush border was prepared from the proximal third of small intestine according to Schmitz et al. [15].

In order to obtain larger quantities of brush border for the chromatographic separation of enzymes, the mucosa from the whole small intestine was scraped off and homogenized according to Forstner et al. [16]. Crude brush border was obtained in the sediment after centrifugation of the homogenate at $4000 \times g$ for 20 min. Purified brush border was then prepared treating this fraction according to Schmitz et al. [15] with minor modifications ($CaCl_2$ in 20 mM, instead of 10 mM, concentration and final centrifugation to sediment the brush border fraction prolonged up to 60 min).

The enzymatic activities of intestinal lumen were measured according to Rhodes et al. [7].

Enzyme assays

The incubation mixture for the assay of amino acyl-β-naphthylamide hydrolase activities contained in a total volume of 100 μl: (1) 0.02 μmol of L-amino acyl-β-naphthylamide, (2) 5 μmol of potassium phosphate buffer at the optimal pH (pH 7.5) when L-leucyl-β-naphthylamide or L-lysyl-β-naphthylamide were used as substrates and 5 μmol of Tris · HCl buffer at the optimal pH (pH 8) when α-L-glutamyl-β-naphthylamide was used as substrate, (3) enzyme solution. For the assay of α-L-glutamyl-β-naphthylamide hydrolase activity the enzyme solution was preincubated at 37 °C for 30 min in 50 mM Tris · HCl buffer, pH 8 and 10 mM Ca^{2+}. Incubation was carried out at 37 °C for 15 and 30 min and the amount of β-naphthylamine liberated was determined by the procedure of Goldbarg and Rutenburg [17].

Peptidase activities were determined according to Auricchio et al. [18]. The incubation mixture contained in a total volume of 100 μl 1 μmol and 0.1 μmol of dipeptides and tripeptides, respectively and 5 μmol of Tris · HCl buffer, pH 8.

γ-Glutamyl transpeptidase activity was assayed according to Auricchio et al. [19].

A unit of enzyme activity hydrolyzes 1 μmol of substrate per min.

All enzymatic activities were proportional to the incubation time and to the

enzyme concentration when the assay mixtures contained 0.25–2 munits of peptidase and 0.05–0.4 munit of L-amino acyl-β-naphthylamidase activity.

Sucrase activity was measured by the method of Auricchio et al. [20].

Protein concentration

Protein concentration was estimated by the method of Lowry et al. [21], with bovine albumin as a standard.

Effect of puromycin, p-hydroxymercuribenzoate and heating on the hydrolysis of L-*phenylalanyl*-L-*alanine*

The effect of puromycin was studied by incubating 0.1 μmol of puromycin, 0.1 μmol of dipeptide and 5 μmol of Tris · HCl buffer pH 8 in the total volume of 100 μl.

The effect of *p*-hydroxymercuribenzoate was studied by preincubation of the enzyme solution at 25 °C for 30 min in the presence of 0.1 mM *p*-hydroxymercuribenzoate.

The effect of heating was studied by preincubation of the enzyme solution at 50 °C for 60 min in 10 mM potassium phosphate buffer, pH 7.

Papain solubilization

The incubation mixture contained 1–5 mg/ml of brush border protein in 50 mM Tris · HCl buffer, pH 7.5, containing 20 mM KCl; for each mg of protein 0.1 mg of cysteine and 0.06 mg of papain were added to the incubation mixture. After 30 min at 37 °C, the incubation mixture was centrifuged at 105 000 × *g* for 60 min and the supernatant was used for gel filtration and chromatography.

Reagents

Substrates and reagents used were described in previous reports [9, 18]. α-Methyl-D-glucopyranoside was obtained from Sigma Chemical Co., St. Louis, Mo., USA; concanavalin A-Sepharose, DEAE-Sephadex and Sephadex G-200 from Pharmacia, Uppsala, Sweden.

RESULTS AND DISCUSSION

The results shown in Table I clearly demonstrate that the hydrolysis of α-L-glutamyl-β-naphthylamide in intestinal mucosa is due mainly to an enzyme of the brush border.

Brush border membranes of acceptable purity were obtained in high yield from rabbit intestine according to the method of Schmitz et al. [15]. About 70 % of the activity of sucrase, which is a marker enzyme of the brush border membrane, was in fact recovered in the brush border fraction (P_2) with 11-fold increase of the specific activity, as compared to that of the total homogenate. Only 20 % of the total sucrase activity was present in the subcellular fraction containing the largest part of other cellular membranes (P_1), without any purification of the enzyme. These results are comparable to those obtained by the same technique in human intestine [15] and by a different method in rats [16].

TABLE I

SPECIFIC ACTIVITIES AND RECOVERIES OF THE ENZYMATIC ACTIVITIES WHICH HYDROLYZE AMINO ACYL-β-NAPHTHYLAMIDE AND SUCROSE IN SUBCELLULAR FRACTIONS OF RABBIT INTESTINE (MEAN VALUES±S.E.M. OF FOUR DIFFERENT PREPARATIONS)

Substrate	Units of enzyme activity per mg of proteins				Recovery			Purification in the brush border**	Recovery in the brush border (corrected for sucrose)***
	Homogenate	P_1*	P_2* (brush border)	S_2*	P_1	P_2 (brush border)	S_2		
Sucrose	0.114±0.019	0.081±0.022	1.20±0.18	0.011±0.002	21.0±3.0	67.8±1.8	7.0±2.9	10.7±0.7	100
L-Leucyl-β-naphthylamide	0.064±0.006	0.062±0.009	0.49±0.14	0.015±0.006	28.1±1.4	48.1±2.2	18.5±2.6	7.8±0.9	71.0±4.5
L-Lysyl-β-naphthylamide	0.032±0.004	0.019±0.004	0.13±0.016	0.022±0.004	17.4±1.7	26.9±2.6	46.8±4.2	4.3±0.7	39.8±4.6
α-L-Glutamyl-β-naphthylamide	0.030±0.003	0.035±0.016	0.23±0.063	0.005±0.002	27.9±3.4	45.7±7.3	10.5±2.5	7.4±1.6	67.6±12.0

* Subcellular fractions obtained according to Schmitz et al. [15].
** Specific activity in the homogenate is equal to 1.
*** Calculated assuming a 100 % recovery of sucrase activity in the brush border.

TABLE II

PAPAIN SOLUBILIZATION OF ENZYME ACTIVITIES FROM BRUSH BORDER

Substrate	Specific activity in the brush border*	Enzyme activity (% of original)**	
		Soluble amount	Sediment
L-Phenylalanyl-L-alanine	1.356±0.187	84 (6)	16 (4)
L-Leucyl-glycyl-glycine	0.615±0.101	94 (6)	20 (4)
Glycyl-L-leucine	0.622±0.054	28 (3)***	8 (1)
L-Leucyl-β-naphthylamide	0.49 ±0.14	95 (6)	15 (4)
L-Lysyl-β-naphthylamide	0.13 ±0.016	87 (3)	8 (3)
α-L-Glutamyl-β-naphthylamide	0.23 ±0.063	84 (3)	10 (3)
Sucrose	1.20 ±0.18	78 (3)	17 (3)

* Mean values ± S.E.M. of four different experiments.

** Mean values and, in parenthesis, number of experiments.

*** The low recovery of glycyl-L-leucine hydrolase activity is due to inactivation of the enzyme during papain digestion.

The enzymatic activity hydrolyzing L-leucyl-β-naphthylamide is known to be due mainly to a brush border peptidase. In fact the yield in the brush border, assuming a 100 % recovery of the sucrase activity in this subcellular fraction, was 70 % with a purification of about 8-fold (Table I).

Similar results were obtained for the enzymatic activity hydrolyzing α-L-glutamyl-β-naphthylamide: about 70 % of the total mucosal enzymatic activity was present in the brush border, and the specific activity in the brush border fraction increased approximately 7 times over the homogenate (Table I). Furthermore, the specific activities for the hydrolysis of the tested amino acyl-β-naphthylamides were, in the intestinal lumen, lower than 1 % of the specific activities of the brush border.

In contrast to these results the L-phenylalanyl-L-alanine and the L-leucyl-glycyl-glycine hydrolyzing activities of rabbit small intestine are mainly located in the cell sap: only about 20 % and 15 %, respectively, of the total mucosal enzymatic activities are located in the brush border [22].

All studied arylamidase and peptidase activities of the brush border were well solubilized by papain, with the only exception of the enzymatic activity hydrolyzing glycyl-L-leucine (Table II).

Gel filtration on Sephadex G-200 of solubilized enzymes resulted in partial separation of the α-L-glutamyl-β-naphthylamide hydrolase activity from the activities hydrolyzing dipeptides, tripeptides and the other amino acyl-β-naphthylamides (Fig. 1).

Three distinct peaks were obtained by chromatography on DEAE-Sephadex A-25 (Fig. 2). (1) A first, not absorbed peak, contained the γ-glutamyl transpeptidase activity. (2) A second, minor peak of dipeptidase activity, eluted at the beginning of the KCl gradient, was present in variable amount in different preparations: the enzyme activity present in this peak was able to hydrolyze dipeptides (L-phenylalanyl-L-alanine; glycyl-L-leucine; L-leucyl-glycine; L-lysyl-L-leucine), but showed no amino acyl-β-naphthylamidase activity and only traces, if any, of tripeptidase activity (tested on L-leucyl-glycyl-glycine and tri-L-phenylalanine). (3) A third, major

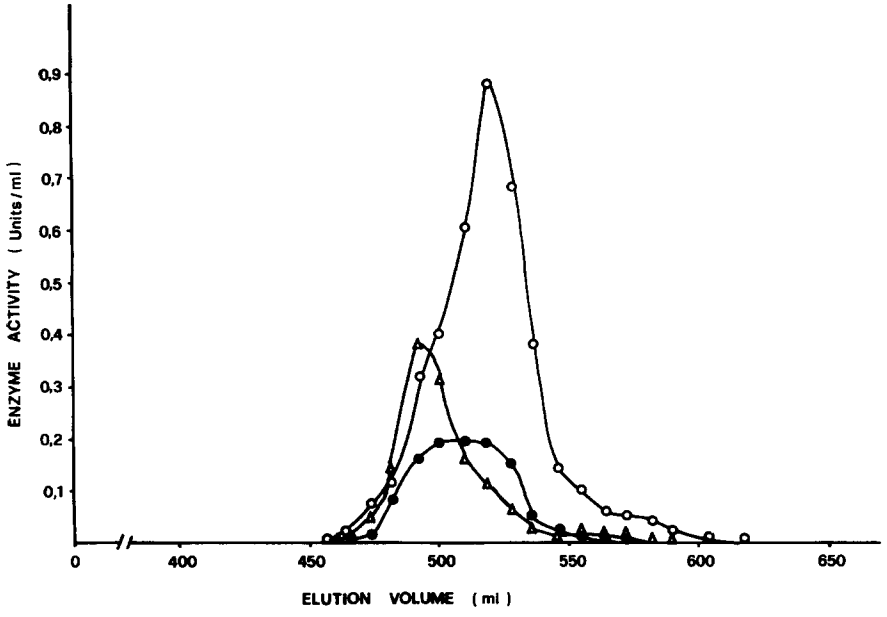

Fig. 1. Elution profile on Sephadex G-200 column (170 cm × 2.5 cm) of the solubilized enzymatic activities of the brush border, hydrolyzing (△—△) α-L-glutamyl-β-naphthylamide, (●—●) L-lysyl-β-naphthylamide and (○—○) L-phenylalanyl-L-alanine. 32 ml of supernatant after papain digestion and centrifugation, containing 8.8 mg protein, were dialyzed against distilled water, lyophilized and dissolved in 1.8 ml of 50 mM Tris·HCl buffer, pH 7.5, containing 20 mM KCl. The sample was applied on the Sephadex G-200 column, equilibrated with the same buffer. The column was developed in the cold room with the same Tris·HCl buffer at a flow rate of 16 ml/h. 4-ml fractions were collected. The enzymatic activities hydrolyzing L-leucyl-β-naphthylamide, glycyl-L-leucine and L-leucyl-glycyl-glycine were eluted together with L-phenylalanyl-L-alanine hydrolase activity and the ratio between them was found to be constant in the various fractions. The peak of γ-glutamyl transpeptidase activity was eluted in the area from 510 to 605 ml.

peak contained all tested amino acyl-β-naphthylamidase and peptidase activities (with the exception of γ-glutamyl transpeptidase activity). When tested on L-phenylalanyl-L-alanine as substrate, the dipeptidase activity of the second peak was not inhibited by puromycin, whereas that of the third peak was inhibited by about 50 %. Furthermore, both enzyme activities were completely resistant to *p*-hydroxymercuribenzoate and partially resistant to heating; these properties were opposite to those of the enzymatic activity of the cytosol, which is inhibited by *p*-hydroxymercuribenzoate and less resistant to heating [22].

An almost complete separation of the α-L-glutamyl-β-naphthylamide hydrolase activity was obtained by chromatography on Sepharose-bound concanavalin A of the third peak eluted from the column of DEAE-Sephadex A-25 (Fig. 3).

Three peaks of enzymatic activities were obtained. The first peak, which was not absorbed, contained all enzymatic activities, but no measurable α-L-glutamyl-β-naphthylamide hydrolase activity and proportionally reduced L-lysyl-β-naphthylamide hydrolase activity. All enzymatic activities, with the only exception of the α-L-glutamyl-β-naphthylamide hydrolase activity, were eluted in a second peak at the beginning of the α-methyl-D-glucopyranoside gradient. α-L-Glutamyl-β-naphthyl-

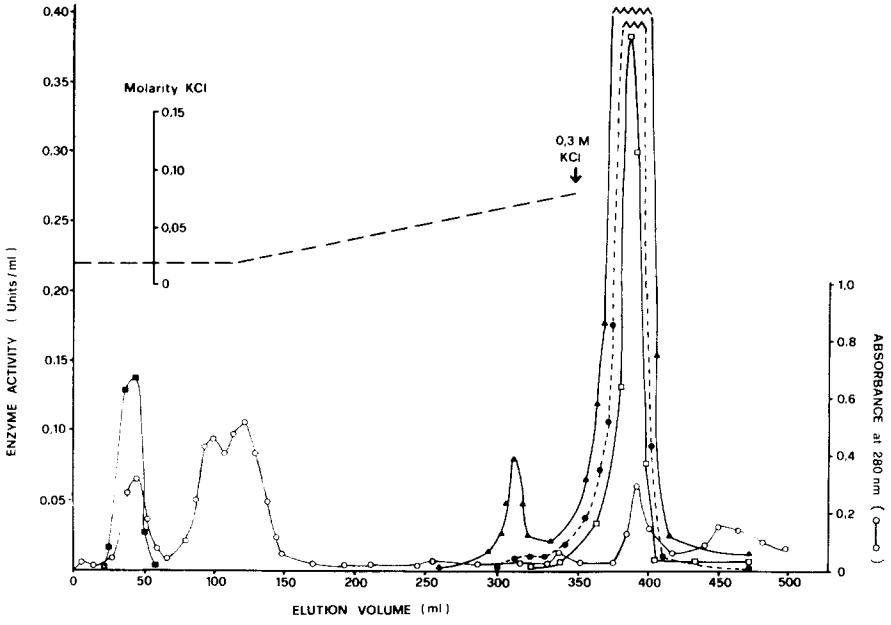

Fig. 2. Chromatographic separation on a DEAE-Sephadex A-25 column (20 cm × 2.3 cm) of the solubilized enzymatic activities of the brush border hydrolyzing (■—■) γ-L-glutamyl-β-naphthylamide, (□—□) L-leucyl-β-naphthylamide, (▲—▲) L-phenylalanyl-L-alanine, (●- - -●) L-leucyl-glycyl-glycine. The activities hydrolyzing α-L-glutamyl-β-naphthylamide and L-lysyl-β-naphthylamide were eluted together with L-leucyl-β-naphthylamide hydrolase activity. 15 ml of supernatant after papain digestion and centrifugation, containing 50 mg protein, were applied on the column. The column was equilibrated with 50 mM Tris·HCl buffer, pH 7.5, containing 20 mM KCl, washed with 120 ml of the same buffer and then developed in the cold-room with a linear gradient of KCl from 20 to 150 mM in the same Tris·HCl buffer (starting buffer 300 ml; final buffer 300 ml). The gradient was interrupted at the beginning of the elution of L-leucyl-β-naphthylamide hydrolase activity (arrow) and the column was then developed with the same Tris · HCl buffer containing 300 mM KCl. The flow rate was 20 ml/h; 4-ml fractions were collected.

amide and L-lysyl-β-naphthylamide hydrolase activities were eluted in a third peak. The specific activity for the hydrolysis of α-L-glutamyl-β-naphthylamide in this last peak was about 40 times higher than that of the homogenate.

In conclusion, the brush border of rabbit intestine contains an enzyme, able to split α-L-glutamyl-β-naphthylamide and separable from the other known major brush border peptidases, the γ-glutamyl transpeptidase and the oligoaminopeptidase. This enzyme is probably similar to (if not identical with) the aminopeptidase A of Glenner et al. [13], which has been already localized in the brush border of rabbit kidney by George and Kenny [14]. The clarification of the role of this intestinal peptidase should await further purification and characterization of the enzyme.

It is interesting to note that α-L-glutamyl-β-naphthylamide hydrolase is very efficiently separated from the oligoaminopeptidase of the brush border by chromatography on concanavalin A-Sepharose, which suggests that this brush border enzyme is a glycoprotein. Since many brush border enzymes are glycoproteins, chromatography on particle-bound lectins could be an useful general method for the purification of these enzymes.

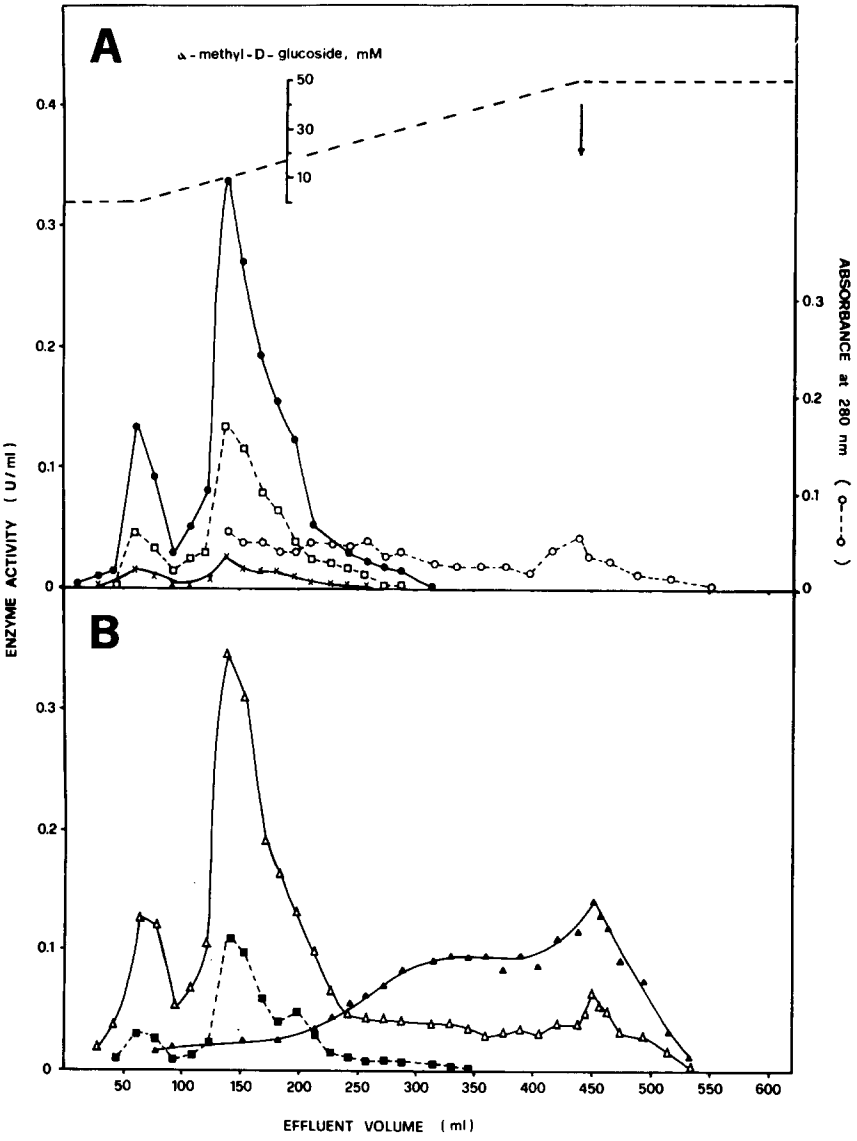

Fig. 3. Chromatographic separation on concanavalin A-Sepharose column (23×1.5 cm) of the last peak of amino acyl-β-naphthylamidase and peptidase activities, obtained by chromatography on DEAE-Sephadex A-25 and containing the enzymic activities hydrolyzing (A) (●—●) L-phenylalanyl-L-alanine, (×—×) glycyl-L-leucine, (□- - -□) L-leucyl-glycyl-glycine, (B) (■—■) L-leucyl-β-naphthylamide, (▲—▲) α-L-glutamyl-β-naphthylamide (10× units), (△—△) L-lysyl-β-naphthylamide (10× units). In the experiment shown in the figure, the third peak from DEAE-Sephadex A-25, containing 12.2 mg of protein, was dialyzed against distilled water, lyophilized, dissolved in 11 ml of 10 mM Tris·HCl buffer, pH 7.5, containing 0.5 mM $CaCl_2$, $MgCl_2$ and $MnCl_2$, and applied on the column equilibrated with the same Tris·HCl buffer. The column was washed in the cold room with 40 ml of the same buffer and then developed with a linear gradient of α-methyl-D-glucopyranoside from 0 to 50 mM in the same buffer (starting and final buffer volumes 200 ml). At the end of the gradient the column was washed with the final buffer. The flow rate was 16 ml/h. 5-ml fractions were collected.

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