Physical and kinetic properties of a plasma-membrane-bound β -D-glucosidase (cellobiase) from midgut cells of an insect (*Rhynchosciara americana* larva)

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1. The midgut caecal cells from *Rhynchosciara americana* larvae possess a plasma-membrane-bound β -D-glucosidase (cellobiase, EC 3.2.1.21), which is recovered (75–95%) in soluble form both after treatment with Triton X-100 and after treatment with papain. 2. The Triton X-100-solubilized β -D-glucosidase displays M_r 106 000 and pI 5.4, whereas the papain-released β -D-glucosidase shows M_r 65 000 and pI 4.7. 3. Thermal inactivations of the detergent-solubilized and the papain-released forms of β -D-glucosidase both follow apparent first-order kinetics with similar half-lives. 4. The papain-released β -D-glucosidase, after being purified by density-gradient centrifugation, hydrolyses β -D-glucosides, β -D-galactosides and β -D-fucosides at the same active site, as inferred from experiments of competition between substrates. 5. The β -D-glucosidase seems to operate in accordance with rapid-equilibrium kinetics, since the K_m (0.61 mM) for the enzyme is constant over a wide range of pH. 6. The hydrolysis of the β -D-glucosidic bond catalysed by the β -D-glucosidase occurs without inversion of configuration, δ -gluconolactone is a strong (K_i 0.5 μ M) inhibitor of the enzyme and substituents in the substrate aglycone affect the catalytic constant of the reaction. These data support the assumption that the mechanism of the reaction catalysed by the β -D-glucosidase involves the intermediary formation of a carbonium ion, rather than a glucosyl-enzyme intermediate.

Digestion in the larvae of the fly *Rhynchosciara americana* starts inside the peritrophic membrane, it follows outside the peritrophic membrane and it ends mainly in the midgut caecal cells (Terra *et al.*, 1979; Terra & Ferreira, 1981). The enzymes that are restricted to the midgut cells occur in major amounts in the plasma membrane. These results led to the proposition that most of the terminal digestion of proteins and carbohydrates, with the exception of trehalose, occurs on the surface of the *R. americana* midgut caecal-cell microvilli (Ferreira & Terra, 1980, 1982). Hence the final stages of digestion in *R. americana* seem to depend on the action of membrane-bound enzymes, as is known to occur in vertebrates (cf. Barnard, 1973).

Although the characterization of the gut plasma-membrane-bound enzymes is a necessary step for a deeper understanding of terminal digestion, thus far such a task has been attempted almost exclusively in

mammals (see review by Kenny & Maroux, 1982). As far as we know, there has hitherto been no study on insect gut membrane-bound hydrolases.

In the present paper we describe some of the physical and kinetic properties of a plasma-membrane-bound β -D-glucosidase (cellobiase, β -D-glucoside glucohydrolase, EC 3.2.1.21) from midgut caecal cells of *R. americana*, which is active on β -D-glucosides, β -D-galactosides and β -D-fucosides.

Experimental

Materials

Acrylamide, bisacrylamide, disaccharides, glucose oxidase (type II), M_r standards, peroxidase (type II) and synthetic substrates were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ampholytes were from Serva Fine Chemicals (Heidelberg, Germany). All the other reagents were of analytical grade from E. Merck (Darmstadt, Germany) and J. T. Baker (Phillipsburg, NJ,

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U.S.A.). The solutions were prepared in double-glass-distilled water.

Animals

Rhynchosciara americana (Diptera: Sciaridae) were reared as described by Lara *et al.* (1965). We used only mature feeding female larvae at the end of the second period of the fourth instar (Terra *et al.*, 1973).

Preparation of midgut caecal plasma membranes

R. americana midgut caeca were homogenized in iso-osmotic saline (0.11 M-KCl, pH 7.0) in an Omni-Mixer (Sorvall) at 15000 rev./min for 20 s. The homogenates, after being filtered through a piece of nylon mesh of 45 μ m pore size, were centrifuged at 25000 *g* for 30 min at 4°C. The sediment, which contains the major part of the plasma membranes (cf. Ferreira & Terra, 1980), was then suspended in the homogenization medium and re-centrifuged at 25000 *g* for 30 min at 4°C.

Solubilization of membrane hydrolases in Triton X-100

Membrane preparations were exposed to Triton X-100 for 20 h at 4°C, in a ratio of 10 mg of Triton X-100/mg of protein, before being centrifuged at 25000 *g* for 30 min. No sediment was visible after the centrifugation of this supernatant at 100000 *g* for 60 min. The activity of the enzymes remains unchanged, at -20°C, for periods of at least a month.

Solubilization of membrane hydrolases in papain

To midgut caecal membranes suspended in 0.1 M-potassium phosphate buffer, pH 7.0, activated (previously incubated with 50 mM-cysteine) papain was added in a ratio of 1 mg of papain to 10 mg of membrane protein. Samples were removed after different periods of time at 30°C, and 7-amino-1-chloro-3-tosylamidoheptan-2-one ('TLCK') was added to them to become in a 20-fold molar excess in relation to papain. The samples were then diluted with water and centrifuged at 100000 *g* for 1 h. The activity of the enzymes remains constant for at least a year at -20°C.

Hydrolase assays and protein determination

Proteins and the hydrolysis of nitrophenol derivatives were determined as previously described (Ferreira & Terra, 1980). The hydrolysis of cellobiose, maltose, salicin and phenyl β -D-glucoside was monitored by measuring the appearance of glucose in the incubation media, by using the glucose oxidase/peroxidase method of Dahlqvist (1968). In each determination, incubations were continued for at least four different periods of time and the initial rates were calculated. All assays were performed

under conditions such that measured activity was proportional to protein and to time.

When δ -gluconolactone was present, initial rates were calculated from incubations conducted for 20 min at 30°C in 100 mM-sodium phosphate buffer, pH 6.2. In these conditions, 32% of the initial concentration of δ -gluconolactone undergoes spontaneous hydrolysis. No lactonase (EC 3.1.1.17) activity occurs in our preparations. The reaction media of the enzymes previously solubilized in Triton X-100 contained at least 0.05% of this detergent.

Polyacrylamide-gel electrophoresis

Samples were applied to polyacrylamide gels prepared as described by Hedrick & Smith (1968). The electrophoretic separation, the immersion of gels in buffer to decrease their concentration in Tris, the fractionation of gels in a gel fractionator and the collection of gel fractions with a fraction collector were performed as described by Terra & Ferreira (1983). When the material submitted to electrophoresis was in detergent, the fractionation buffer contained 0.1% Triton X-100. The recoveries of the activities applied to the gels were approx. 77%.

Isoelectric focusing in polyacrylamide gels

Isoelectric focusing was performed as described by Terra *et al.* (1978), in columns of 7.5% polyacrylamide gels containing a mixture of the ampholytes pH 4-6 and pH 5-7 (1% of each ampholyte), after a pre-focusing of 30 min at 31 V/cm. When samples solubilized in detergent were to be focused, the gels also contained 0.17% Triton X-100. The recoveries of the activities applied to the gels were approx. 74%.

Density-gradient centrifugation

Samples (0.2 ml) of membrane proteins solubilized with Triton X-100 or papain, containing 1.5 mg of bovine haemoglobin and 50 μ g of bovine liver catalase, were layered on the top of 4.6 ml linear glycerol gradients (5-30%, w/v) made up in 50 mM-sodium phosphate buffer, pH 6.2. The centrifugations and the collection of fractions were performed as described previously (Terra & Ferreira, 1983). The M_r values of the enzymes assayed in the fractions were calculated by the method of Martin & Ames (1961), with as reference standards the sedimentation rates of bovine haemoglobin (M_r 64 500) and bovine liver catalase (M_r 232 000). The recoveries of the activities applied to the gradients were approx. 100%.

Competition of cellobiose and p-nitrophenyl β -D-glucoside for β -D-glucosidase

In this experiment two samples were taken from each reaction medium at the end of the incubation.

In one sample *p*-nitrophenol was determined, from which the amount of *p*-nitrophenyl β -D-glucoside hydrolysed was calculated; in the other sample glucose was measured, by the method of Dahlqvist (1968). After allowance for the amount of glucose originating from *p*-nitrophenyl β -D-glucoside, it was possible to calculate the activity on cellobiose.

Configuration of the anomeric carbon atom of reaction products

In order to identify the anomeric configuration of the D-glucose produced by the β -D-glucosidase (by a method based on that of Semenza *et al.*, 1969), it was incubated for 15 min at room temperature (23°C) in 20 mM-sodium acetate buffer, pH 5.6, with *o*-nitrophenyl β -D-glucoside as substrate. After 15 min, the cellobiase reaction was stopped by the addition of δ -gluconolactone (1 mM final concentration) to the incubation medium, from which was removed one 0.1 ml sample before and another after boiling of the medium for 5 min. To each of the samples, 1 ml of the glucose oxidase/peroxidase reagent (Dahlqvist, 1968) was added and the absorbance was recorded continuously at 420 nm. Controls were prepared similarly to the experimental samples, with the substrate replaced by a fresh solution of α -D-glucose or by a boiled solution of α -D-glucose, the glucose concentrations being the

same as those released from the glucoside in the experimental samples. The controls were incubated for 7.5 min before the addition of δ -gluconolactone and the glucose oxidase/peroxidase reagent. Aldose 1-epimerase (mutarotase, EC 5.1.3.3) is absent from our preparations.

Results

Solubilization of the β -D-glucosidase by treatment with detergent and with papain

About 85% of the activity originally present in the midgut caecal membranes of *R. americana* is solubilized in Triton X-100, and about 75% of the original activity is released into solution by 45–60 min of treatment with papain. In each case, the solubilized activity and the non-solubilized activity add up to $95 \pm 5\%$ of the activity found originally in the preparation of membranes.

M_r and pI of the β -D-glucosidase

Isoelectric focusing (Fig. 1), density-gradient centrifugation (Fig. 2) and polyacrylamide-gel electrophoresis (Fig. 3) suggest that there is only one molecular species of the β -D-glucosidase, both in detergent-treated and in papain-treated preparations. Furthermore, the results suggest that the β -D-glucosidase hydrolyses, besides cellobiose,

Fig. 1. Isoelectric focusing of solubilized midgut caecal hydrolases

Samples solubilized in Triton X-100: (a) assay with *p*-nitrophenyl β -D-galactoside; (b) assay with cellobiose. Samples solubilized by treatment with papain: (c) assay with *p*-nitrophenyl β -D-galactoside; (d) assay with cellobiose. O, A_{420} ; ●, pH. Profiles obtained from several other preparations are similar to those shown, which were obtained from the same experiment (gel). Details are given in the Experimental section.

Fig. 2. Sedimentation profiles of solubilized midgut caecal hydrolases in a linear glycerol gradient. Fractions (0.2 ml) were collected from the bottom of the tube. Samples solubilized in Triton X-100: (a) assay with *p*-nitrophenyl β -D-galactoside; (b) assay with cellobiose; (c) assay with *p*-nitrophenyl β -D-fucoside. Samples solubilized by treatment with papain: (d) assay with *p*-nitrophenyl β -D-galactoside; (e) assay with cellobiose; (f) assay with *p*-nitrophenyl β -D-fucoside. M_r markers: C, bovine liver catalase (M_r 232 000); H, bovine haemoglobin (M_r 64 500). Profiles obtained from several other preparations are similar to those shown, which were obtained from the same experiment (gradient). Details are given in the Experimental section.

p-nitrophenyl β -D-glucoside, *p*-nitrophenyl β -D-galactoside and *p*-nitrophenyl β -D-fucoside. This is supported by the electrophoretic profiles for pooled fractions of the papain-released form of the β -D-glucosidase obtained by centrifugation (Fig. 4). The detergent-solubilized form of the β -D-glucosidase has pI 5.4 (Fig. 1) and M_r $106\,400 \pm 6600$ (Fig. 2), whereas the papain-released form has pI 4.7 (Fig. 1) and M_r $64\,834 \pm 6000$ (in the absence of detergent) (Fig. 2) or $72\,100 \pm 10\,700$ (in the presence of detergent).

Thermal inactivation of the β -D-glucosidase

Thermal inactivation of both the detergent-solubilized and the papain-released forms of the β -D-glucosidase follows apparent first-order kinetics with similar half-lives (17 min and 14 min respectively), for a period of time of at least 3 half-lives.

Effects of pH and temperature on the β -D-glucosidase

The effect of pH on the β -D-glucosidase (papain-released form) activity suggests the existence of a protonated (pK_a 7.7) group in the active site of the functional form of the enzyme, which shows an apparent optimum pH at approx. 6.2 (50 mM-citrate/phosphate buffer) (Fig. 5). The changes in the curves at pH 6.75 correspond to a change of buffer and seem to be a consequence of their different components and ionic strength. The use of an *NNN'*-tetramethylethylenediamine/HCl buffer, instead of citrate/phosphate and pyrophosphate buffers, results in a smooth curve of the plot of $\log v$ against pH. Nevertheless, since tetramethylethylenediamine is a competitive inhibitor for β -D-glucosidase (K_i 183 mM at pH 7.5), it affects the other

Fig. 3. *Electrophoretic separation of solubilized midgut caecal hydrolases in 9%-polyacrylamide-gel columns* Samples solubilized in Triton X-100: (a) assay with *p*-nitrophenyl β -D-galactoside; (b) assay with cellobiose. Samples solubilized by treatment with papain: (c) assay with *p*-nitrophenyl β -D-galactoside; (d) assay with cellobiose. Profiles obtained from several other preparations are similar to those shown, which were obtained from the same experiment (gel). Details are given in the Experimental section.

Table 1. *Substrate specificity of the purified midgut caecal β -D-glucosidase*

Purified β -D-glucosidase (see the legend to Fig. 4) was incubated with five different concentrations of each of the listed carbohydrates, in 50 mM-phosphate buffer, pH 6.2, at 30°C. Kinetic parameters (means $\pm$ s.d.) were determined by a weighted linear regression by the procedure of Wilkinson (1961) with the aid of a programmable pocket calculator (Texas Instruments T159). Methyl β -D-glucoside was also tested, but was found not to be hydrolysed by the enzyme.

Substrate	K_m (mM)	V (nmol $\cdot$ s $^{-1}$)	$10^4 \times V/K_m$ (s $^{-1}$)
Cellobiose	0.61 ± 0.08	252 ± 7	4.13 ± 0.66
<i>o</i> -Nitrophenyl β -D-glucoside	0.90 ± 0.17	230 ± 13	2.55 ± 0.63
<i>p</i> -Nitrophenyl β -D-glucoside	0.53 ± 0.09	167 ± 7	3.15 ± 0.67
<i>p</i> -Nitrophenyl β -D-fucoside	0.50 ± 0.03	175 ± 10	3.51 ± 0.40
Salicin	0.80 ± 0.12	8.5 ± 0.3	0.106 ± 0.020
<i>p</i> -Nitrophenyl β -D-galactoside	5.3 ± 0.7	251 ± 20	0.47 ± 0.10
Phenyl β -D-glucoside	3.5 ± 0.5	6.5 ± 0.4	0.019 ± 0.004

curves in a complex way. The results also show that K_m is pH-independent (Fig. 5).

Results obtained with the detergent-solubilized form of the β -D-glucosidase were essentially the same as those illustrated (Fig. 5) for the papain-released form.

The energy of activation of the midgut β -D-glucosidase determined in saturating conditions between 20 and 48°C is 53.6 kJ/mol (12.8 kcal/mol). The enzyme is completely stable for at least 2 h in 50 mM-phosphate buffer, pH 6.2, at temperatures up to 40°C.

Substrate specificity and inhibitors of the purified β -D-glucosidase

Tris (K_i 19 mM), δ -gluconolactone (K_i 0.5 μ M) and γ -galactonolactone (K_i 0.3 mM) are simple linear competitive inhibitors of the β -D-glucosidase. Gluconic acid does not inhibit the enzyme even in concentrations 50-fold (0.1 mM) higher than those for δ -gluconolactone.

The purified β -D-glucosidase (Fig. 4) hydrolyses β -D-glucosides, β -D-galactosides and β -D-fucosides (Table 1), and does not hydrolyse α -D-glucosides

Fig. 4. Electrophoresis of purified midgut caecal β -D-glucosidase

The β -D-glucosidase was purified by density-gradient centrifugation of the enzyme solubilized by treatment with papain, followed by pooling fractions equivalent to fractions 17, 18 and 19 of the gradient (e) of Fig. 2 from several gradients. (a) Assay with *p*-nitrophenyl β -D-galactoside; (b) assay with cellobiose; (c) assay with *p*-nitrophenyl β -D-fucoside. Profiles obtained from several other preparations are similar to those shown, which were obtained from the same experiment (gel). Details are given in the Experimental section.

Table 2. Inhibition by glycosides of the purified midgut caecal β -D-glucosidase

Purified β -D-glucosidase (see the legend to Fig. 4) was incubated with each of the listed carbohydrates (15 mM) with and without cellobiose (3 mM) in 50 mM-phosphate buffer, pH 6.2, at 30°C. None of the carbohydrates listed has substrate properties, although in various degrees they affected the action of the enzyme on cellobiose. Values are means $\pm$ S.D. for three independent determinations.

Carbohydrate	Inhibition (%)
Trehalose	0.0
Melibiose	20.2 $\pm$ 3.8
Maltose	44.8 $\pm$ 7.9
<i>p</i> -Nitrophenyl α -D-glucoside	48.6 $\pm$ 3.2
Sucrose	51.0 $\pm$ 5.0

Fig. 5. Effect of pH on the stability and on some kinetic parameters of the papain-released form of the midgut caecal β -D-glucosidase

The enzyme samples were incubated in the different pH values at several cellobiose concentrations, and the apparent V and apparent K_m values were extrapolated from Lineweaver-Burk plots. The assays were performed at 30°C in 50 mM-citrate/phosphate buffer (O) and 50 mM-pyrophosphate buffer (●). For the determination of the pH-stability, the enzyme was left for 2 h at 30°C at different pH values, before being diluted 10-fold by the addition of 0.1 M-phosphate buffer, pH 6.2, followed by assays in these conditions. Units: V , $\mu\text{M}\cdot\text{s}^{-1}$; K_m , mM; V/K_m , 10^3s^{-1} .

and α -D-galactosides (Table 2). Cellobiose and *p*-nitrophenyl β -D-glucoside seem to be hydrolysed at the same active site, since each of these two substrates is a simple linear competitive inhibitor in the reaction of the β -D-glucosidase on the other (Fig. 6). Furthermore, the K_i values for *p*-nitrophenyl β -D-glucoside (0.40 mM) and for cellobiose (0.73 mM) agree with their respective K_m values (0.53 and 0.61 mM) (Table 1).

The experiments involving competition between substrates with the purified β -D-glucosidases (Table

Fig. 6. Mutual competition of substrates of the purified midgut caecal β -D-glucosidase: *p*-nitrophenyl β -D-glucoside (*pNPβGlu*) inhibition of cellobiose hydrolysis and cellobiose inhibition of *p*-nitrophenyl β -D-glucoside hydrolysis. Lineweaver-Burk plots for different concentrations of the inhibitor substrate are shown; the inset shows a replot of slopes from Lineweaver-Burk plots against the concentration of the inhibitor substrate. Details of the purification of the β -D-glucosidase are given in the legend to Fig. 4.

Table 3. Mixed-substrate analysis: calculated and observed reaction rates of the purified midgut caecal β -D-glucosidase. Substrates A and B were added together and incubated with purified β -D-glucosidase (see the legend to Fig. 4) in 50 mM-phosphate buffer, pH 6.2, at 30°C. The observed v values are the means $\pm$ s.d. for three independent determinations. The values calculated for a reaction due to two sites were obtained by a simple summation of the hydrolytic rate of each substrate and the values calculated assuming only one site determined by the method of Walker & Axelrod (1978).

Substrate A (10 mM)	Substrate B (10 mM)	Calculated v ($\text{nm} \cdot \text{s}^{-1}$)		Observed v ($\text{nm} \cdot \text{s}^{-1}$)
		One site	Two sites	
<i>p</i> -Nitrophenyl β -D-glucoside	<i>p</i> -Nitrophenyl β -D-galactoside	87.2	123	70.6 ± 6.5
<i>p</i> -Nitrophenyl β -D-glucoside	<i>p</i> -Nitrophenyl β -D-fucoside	52.3	124	46.9 ± 1.2

3) also suggest that it is able to hydrolyse β -D-glucosides, β -D-galactosides and β -D-fucosides at the same site. In addition, the K_i for γ -galactonolactone with *p*-nitrophenyl β -D-galactoside as substrate is identical with that obtained with cellobiose as substrate.

Anomeric configuration of the D-glucose liberated by the purified β -D-glucosidase

The purified midgut β -D-glucosidase liberates β -D-glucose from *o*-nitrophenyl β -D-glucoside (Fig. 7), suggesting that the reaction catalysed by the enzyme occurs with retention of configuration. The

same result is obtained with the detergent-solubilized form of the midgut β -D-glucosidase.

Discussion

Physical-chemical aspects of the midgut β -D-glucosidase

The *R. americana* midgut caecal β -D-glucosidase was shown, by several criteria, to be bound to the plasma membrane covering the cellular microvilli (Ferreira & Terra, 1980, 1982). In the present paper it is shown that the enzyme is solubilized from the midgut caecal membranes by treatment with Triton

Fig. 7. Enzymic identification of the anomeric configuration of the D-glucose liberated by purified midgut caecal β -D-glucosidase

Curve A, α -D-glucose control; curve B, α -D-glucose control after anomerization (boiling); curve C, D-glucose liberated by the β -D-glucosidase (after anomerization, the experimental curve becomes similar to curve B). The concentration of glucose and other experimental conditions for curves A and B are identical with those for curve C. Purified β -D-glucosidase was as indicated in the legend to Fig. 4; other details are given in the Experimental section.

X-100 and with papain. Hence the evidence suggests that the *R. americana* midgut β -D-glucosidase is an integral protein of the plasma membrane of the midgut caecal cells.

Papain treatment decreases the M_r of the midgut β -D-glucosidase from 106 000 (measured in the presence of detergent) to 65 000 (72 100 if measured in the presence of detergent), without changing the catalytic parameters of the enzyme or its stability in relation to pH or temperature. This suggests that papain (in accordance with the findings obtained by several other authors with other systems; see review by Kenny & Maroux, 1982) separates a catalytic and hydrophilic domain of the β -D-glucosidase from a non-catalytic and presumably hydrophobic domain, which might be responsible for the insertion of the enzyme into the microvillus plasma membrane.

Specificity, inhibition and mechanism of the midgut β -D-glucosidase

The papain-released form of the β -D-glucosidase hydrolyses β -D-glucosides, β -D-galactosides and β -D-fucosides, but does not hydrolyse α -D-glucosides. Attempts to resolve the activity on cellobiose from the activity towards other β -D-glycosides by using

several separation techniques (isoelectric focusing, polyacrylamide-gel electrophoresis and density-gradient centrifugation) were unsuccessful. Furthermore, experiments with purified β -D-glucosidase involving competitive substrate inhibition suggested that β -D-glucosides, β -D-galactosides and β -D-fucosides were hydrolysed at the same active site. These results support the view that the *R. americana* midgut β -D-glucosidase is a β -D-glycosidase with a wide substrate specificity.

The K_m of the β -D-glucosidase is pH-independent (Fig. 5), which suggests (cf. Tipton & Dixon, 1979) that the enzyme exhibits rapid-equilibrium kinetics.

The mechanism of the reaction catalysed by the *R. americana* midgut β -D-glucosidase might involve the intermediary formation of a carbonium ion, which should be attacked by water from the front side, from consideration of the following: (a) the hydrolysis of the β -D-glucoside bond occurs without inversion of configuration; (b) the rate-limiting step in the reaction seems to be the departure of the aglycone, since substituents in the aglycone do influence the catalytic constant of the β -D-glucosidase; (c) δ -gluconolactone (but not gluconic acid) is a powerful inhibitor of the enzyme, which suggests that the lactone (similar in structure to the carbonium ion; cf. Levvy & Snaith, 1972) is an analogue of the transition state formed during β -D-glucosidase catalysis (cf. Wolfenden, 1972; Lienhard, 1973).

γ -Galactonolactone inhibits the *R. americana* midgut β -D-glucosidase with a K_i 10-fold lower than the K_m for any of the β -D-galactosides. This suggests that the β -D-galactosides are hydrolysed by the midgut β -D-glucosidase by a mechanism similar to that discussed above for β -D-glucosides, and is in accordance with the view that β -D-galactosides are hydrolysed at the same active site that hydrolyses β -D-glucosides.

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