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Inhibition of a model protease — pyroglutamate aminopeptidase by a natural oligosaccharide gum from *Hakea gibbosa*

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Abstract

The purpose of this study was to investigate the effect of the oligosaccharide gum from *Hakea gibbosa* on the activity of a model protease enzyme pyroglutamate aminopeptidase (5-oxoprolyl peptidase; EC 3.4.19.3) and to elucidate the mechanism responsible for the decreased activity. Enzyme kinetic studies were conducted at 37°C in 100 mM potassium phosphate buffer with 10 mM EDTA, 5% (v/v) glycerol, and 5 mM DTT (pH 8) for 15 min and were performed both in the presence and absence of the gum. Enzymatic activity was determined by a colorimetric assay using the specific substrate L-pyroglutamic acid β-napthylamide. The enzyme kinetics was studied at various substrate and gum concentrations. The velocity of the reaction was determined by the amount of the product (β-napthylamine) liberated at each substrate and gum concentration. The K_s and V_{max} of the enzyme in the absence of the gum were 24.40 ± 2.14 μM and 502.95 ± 28.90 nmoles·min^{-1}·mg protein^{-1}, respectively. As the concentration of the gum was gradually increased from 0.1 to 2%, the value of the V_{max} decreased from 318.94 ± 21.46 to 158.83 ± 24.51 nmoles·min^{-1}·mg protein^{-1} while K_s increased from 17.42 ± 4.6 to 63.03 ± 1.89 μM. The mechanism for the inhibition of the enzyme by Hakea appeared to be a mixed-linear type (a type of non-competitive inhibition) as suggested from Hanes-Woolf, Dixon and Cornish-Bowden plots. The turnover number, k_{cat}, calculated for the enzyme also decreased from 14.09 ± 0.81 to 4.45 ± 0.69 min^{-1} as the concentration of the inhibitor was incrementally increased from 0 to 2% (w/v). The K_i and αK_i calculated from Dixon and Cornish-Bowden plots were found to be 0.31 ± 0.11% (w/v) and 1.33 ± 0.42% (w/v), respectively. The natural gum from *Hakea gibbosa* was effective in non-competitively inhibiting the enzyme pyroglutamate aminopeptidase. Thus, the natural gum may be a promising additive not only for its sustained-release and mucoadhesive properties as shown previously, but also for its ability to slow the enzymatic degradation of therapeutic polypeptides incorporated in dosage forms.

Keywords: Enzyme kinetics; Non-competitive inhibition; Pyroglutamate aminopeptidase; Dixon plot; Hanes-Woolf plot; Cornish-Bowden plot

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Although possible alternate routes of protein and peptide delivery are being investigated vigarously, the oral route of protein and peptide administration still offers the greatest ease of application. However, the oral route of protein and peptide administration has met with limited success due to a variety of reasons (Harris and Robinson, 1990; Lang et al., 1998), including (a) low permeability due to hydrophilicity, globular structure, and size, (b) inactivation by the gastric acid and luminal enzymes at the site of absorption before reaching the systemic circulation, and (c) short residence time of the drug as well as the delivery device at the site of absorption. All these factors lead to sub-therapeutic plasma concentrations rendering the protein or peptide clinically ineffective. Attempts have been made to overcome these barriers by using enzyme inhibitors but little success has been realized.

Mucoadhesive polymers have been employed not only to control protein and peptide release from a delivery device, but also to prolong the residence time at the site of drug absorption, and increase the contact time between the delivery device and the absorbing mucosa. Recently, the novel concept of using mucoadhesive polymers as enzyme inhibitors has been developed for peroral drug delivery. Mucoadhesive polymers such as polycarbophil (Noveon® AA1), carbomer (Carbopol® 934P), chitosan (Daichitosan® VH), chitosan glutamate (SeaCure® G210), chitosan hydrochloride (chitosan-HCl) have shown the ability to decrease the extent of enzymatic degradation of peptides such as 9-desglycinamide, 8-arginine vasopressin (DGAVP) (Lueßen et al., 1994), buserelin (Lueßen et al., 1994), human luteinizing hormone releasing hormone (LHRH) (Walker et al., 1999), insulin (Bai et al., 1995), calcitonin (Bai et al., 1995), and insulin-like growth factor (Bai et al., 1995). In addition, these mucoadhesive polymers increase the paracellular transport by opening the intestinal intercellular junctions (Lueßen et al., 1994; Bai et al., 1995). Polymers such as Carbopol® 934P and Noveon® AA1 by virtue of their ability to chelate various divalent metal ions such as Ca^{2+} and Zn^{2+} inhibit many of the intestinal metalo exo- and endopeptidases but were found not to inhibit pyroglutamate aminopeptidase in vitro (Lueßen et al., 1996a,b).

Pyroglutamate aminopeptidase (molecular weight 28 kDa) (Bauer, 1988) is a pharmaceutically important proteolytic enzyme responsible for the degradation of several bioactive proteins and peptides containing an amino terminal pyroglutamyl residue including thyrotropin-releasing hormone (TRH) (Moss and Bundgaard, 1989), luteinizing hormone releasing hormone (LHRH) (Moss and Bundgaard, 1989), neurotensin (Bauer, 1988), gastrin (Moss and Bundgaard, 1989), collagen (Podell and Abraham, 1978), and immunoglobulins (Podell and Abraham, 1978).

Our previous studies have shown the ability of a natural gum from *Hakea gibbosa* to not only sustain the delivery of a variety of molecules such as chlorpheniramine maleate (Alur et al., 1999, 1999a) and salmon calcitonin (Alur et al., 1999b), but also its ability to adhere to mucosa, both, in vitro and in vivo. Hakea gum is a polysaccharide exudate from the tree *H. gibbosa* (Fam: Proteaceae). The gum has a molecular weight of greater than 2×10^3 kDa and the composition and the chemical structure of the gum has been provided in our previous reports (Alur et al., 1999a; Eagles, 1992). Thus, the aim of the present study was to further evaluate the utility of the natural gum by investigating the effect of this gum on the activity of the enzyme pyroglutamate aminopeptidase (5-oxoprolyl peptidase; EC 3.4.19.3) in vitro.

The Hakea gum was a gift from Dr Peter Eagles of the University of the Western Cape, Cape Town, South Africa. Pyroglutamate aminopeptidase (a calf liver preparation) (EC 3.4.19.3), L-pyroglutamic acid β-napthylamide, potassium phosphate (dibasic, trihydrate), ethylenediaminetetraacetic acid (EDTA) (disodium salt, dihydrate), glycerol, DL-dithiothreitol (DTT), methanol (absolute), trichloroacetic acid (TCA) (6.1 N solution), sodium nitrite tablets (2 mg), 0.5% w/v ammonium sulfamate solution, ethyl alcohol (denatured), *N*-1-naphthylethylenediamine, β-naphthylamine standard solution (LAP calibration solution) were obtained from Sigma chemical company (St Louis, MO). All solutions were prepared in deionized distilled water, unless mentioned. All other materials, except for the gum, were used as received. The gum was

purified by first dissolving it in water and then filtering the 2% (w/v) solution through muslin cloth. The filtered solution was freeze-dried using a model 10-MR SA Virtis table top freeze drier (Gardiner, NY).

The enzyme kinetic studies were conducted at 37°C in 100 mM potassium phosphate buffer (pH adjusted to 8.0 with 1N HCl or 1N NaOH) with 10 mM EDTA, 5% (v/v) glycerol, and 5 mM DTT for 15 min with 0, 0.1, 0.25, 0.75, 1.25, and 2% (w/v) of the gum. Pyroglutamate aminopeptidase enzyme solution was prepared in the above-mentioned ice-cold buffer (without gum) immediately prior to use, containing 35–70 units ml^{-1} of the enzyme. Stock solutions (1.25, 2.5, 5, 10, and 20 mM) of L-pyroglutamic acid β-naphthylamide (substrate) were prepared in absolute methanol. The enzyme assay procedure was supplied by Sigma and was followed without further modification. To a test solution containing 1 ml of the gum-buffer solution, 0.1 ml of the substrate solution was added and equilibrated at 37°C. Next, 0.1 ml of the enzyme solution was added and incubated at 37°C for 15 min. Following the incubation period, 1 ml 25% (v/v) TCA solution was added to arrest the reaction. A blank solution was prepared by adding 0.1 ml substrate solution to 1 ml gum-buffer solution and then equilibrating at 37°C. This solution was also incubated for 15 min at 37°C. One mililitre 25% (v/v) TCA solution was added, followed by 0.1 ml of the enzyme solution at the end of 15-min incubation period. The velocity of the reaction was determined by the amount of the product (β-naphthylamine) liberated at each substrate and gum concentration by a diazotization reaction. To 1 ml test and blank solution, 1 ml 0.1% (w/v) sodium nitrite solution was added and the mixture was incubated at room temperature for 3 min. The incubation was followed by the addition of 1 ml 0.5% (w/v) ammonium sulfamate and incubation at room temperature for another 3 min. Finally, 2 ml 0.05% (w/v) *N*-1-naphthylethylenediamine solution was added and incubated at room temperature for 45 min. The absorbance of the samples was recorded at 580 nm at the end of 45-min incubation period. The amount of β-naphthylamine liberated in the test samples was determined from a calibration curve constructed using a standard solution of 0.0018% (w/v) β-naphthylamine. After each addition, the solution was vortexed for 5 min.

To estimate the values of the kinetic parameters of saturable kinetics, the velocity of the reaction was modeled to the following equation using a nonlinear least-squares regression analysis program (WIN NONLIN®):

$$v = \frac{v_{max} C}{K_S + C}, \quad (1)$$

where v_{max} is the maximum velocity of the reation, C is the substrate concentration, and K_s is the half-saturation concentration (Michealis–Menten constant).

All experiments were performed in triplicate and the results were expressed as the mean value ± the standard deviation. Mean values were tested for statistical significance by using analysis of variance (ANOVA). A Bonferroni's post hoc test was used to identify mean differences if a significant F value was calculated ($P < 0.05$). It is evident from Fig. 1 that as the percent of hakea was gradually raised in the buffer, the velocity of

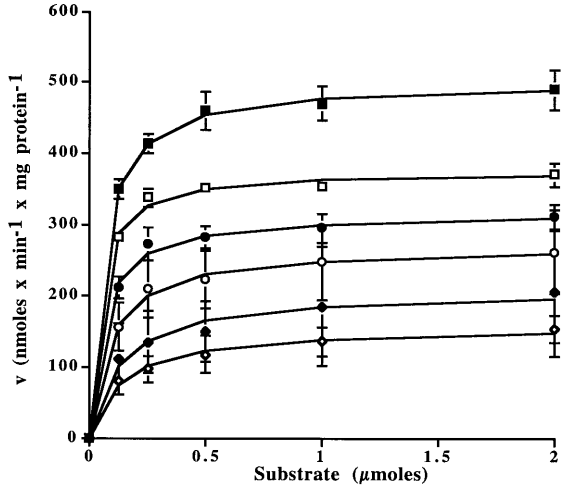


Fig. 1. Typical enzyme kinetic plot of initial velocity against L-pyroglutamic acid β-napthylamide concentration in the absence (–■–) and presence of 0.1% (–□–), 0.25% (–●–), 0.75% (–O–), 1.25% (–◆–), and 2% (–◇–) of hakea. All data points represent the mean value ± standard deviation of three experiments. Lines through mean values represent a mathematical fit of the data.

Table 1
Kinetic parameters of pyroglutamate aminopeptidase

Hakea (%)	v_{max} (nmoles·min^{-1}·mg protein^{-1})	K_s (μM)	k_{cat} (min^{-1})	Time for one catalytic cycle (s)
0	502.95 ± 28.90	24.40 ± 2.14	14.09 ± 0.81	4.27 ± 0.24
0.1	377.83 ± 4.57	17.42 ± 4.60	10.58 ± 0.13	5.67 ± 0.07
0.25	318.94 ± 21.46[a]	25.76 ± 4.22	8.93 ± 0.60	6.74 ± 0.4
0.75	272.17 ± 63.41[a]	39.70 ± 21.63	7.62 ± 1.78	8.18 ± 2.03
1.25	211.32 ± 91.27[a]	58.48 ± 12.41[b]	5.92 ± 2.56	11.30 ± 4.09[c]
2	158.83 ± 24.51[a]	63.03 ± 1.89[b]	4.45 ± 0.69	13.69 ± 1.98[c]

[a] Indicates a statistically significantly lower value of v_{max} in the presence of hakea as compared to the corresponding value in the absence of hakea.

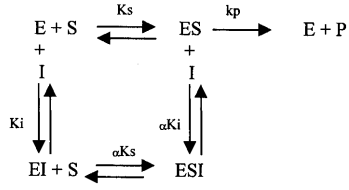
[b] Indicates a statistically significantly greater value of K_s in the presence of hakea as compared to the corresponding value in the absence of hakea.

[c] Indicates a statistically significantly greater value of the time for one catalytic cycle in the presence of hakea as compared to the corresponding value in the absence of hakea.

the reaction decreased. Kinetic parameters such as V_{max} and K_s ($= K_M$) were determined from the fit and are listed in Table 1. The time for one catalytic cycle of the enzyme was also calculated from the turnover number (k_{cat}, k_p) (Segel, 1993) of the enzyme and these values are also listed in Table 1. As the concentration of the gum was gradually increased, the mean values for V_{max} decreased (Table 1), while the mean values for K_s increased (Table 1). The mean values for k_{cat} also decreased with a gradual increase in the concentration of the gum.

Enzymes usually exhibit an optimum pH at which the activity of the enzyme is greatest. An increase or decrease in the pH of the reactive medium may cause potential inactivation of an enzyme and hence a decrease in the reaction velocity (Segel, 1993a). The mean value of pH measured at various concentrations of hakea did not vary considerably (mean value of pH was 8.05 ± 0.03) indicating that the enzyme was not inactivated by a change in reactive media pH in the present study.

The mechanism of inhibition seemed to be of mixed-type (a type of noncompetitive inhibition) from the Hanes-Woolf plot (Fig. 2) since the control and 'plus inhibitor' plots intersected below the substrate concentration axis. The equilibria of this type of a system (Segel, 1993a) may be illustrated as:



where, K_s, K_i, αK_s, and αK_i represent the various equilibrium constants for enzyme-substrate (ES), enzyme-inhibitior (EI), enzyme-substrate-in-

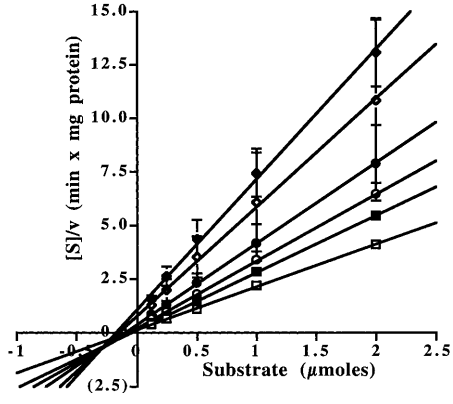


Fig. 2. Hanes-Woolf plot of [S]/v against [S] in the absence (□, $r^2 = 0.999$) and presence of 0.1% (■, $r^2 = 0.999$), 0.25% (O, $r^2 = 0.999$), 0.75% (●, $r^2 = 0.999$), 1.25% (◇, $r^2 = 0.998$), and 2% (◆, $r^2 = 0.997$) of hakea. All data points represent the mean value ± standard deviation of three experiments. Lines through mean values represent a mathematical fit of the data using a linear least-squares regression analysis.

Table 2
Kinetic constants estimated from replots by various methods

Method	αK_i (%)	K_i (%)	αK_s (μmoles)	α	K_i' (%)
Hanes-Woolf replot	0.80 ± 0.30[a]	0.27 ± 0.13	–	3.24 ± 0.45	–
Cornish-Bowden replot	–	–	0.30 ± 0.05	5.55 ± 0.92[b]	–
Cornish-Bowden plot	–	–	–	–	1.33 ± 0.42
Dixon plot	–	0.31 ± 0.11	–	–	–

[a] Indicates a statistically significantly greater value of αK_i as compared to the value of K_i obtained from the Hanes-Woolf replot.
[b] Indicates a statistically significantly greater value of α obtained from the Cornish-Bowden replot as compared to the value of α obtained from the Hanes-Woolf replot.

hibitor (ESI, forms when substrate binds to the EI complex), and enzyme-substrate-inhibitor (ESI, forms when inhibitor binds to the ES complex), respectively. The variable k_p is the rate constant for the breakdown of enzyme-substrate complex to enzyme and product. The variables α and β represent the factor by which K_s and k_p change, respectively when inhibitor occupies the enzyme active site. A mixed-type inhibition affects the values of both V_{max} and K_s of an enzyme catalyzed reaction (Segel, 1993a). V_{max} decreases as the enzyme in the presence of the inhibitor exists in a nonproductive enzyme-substrate-inhibitor (ESI) form, i.e. (ESI) is catalytically inactive (Segel, 1993a). The value of K_s increases since a portion of the enzyme available for combination with substrate will exist in the enzyme-inhibitor (EI) form. This EI form has a lower affinity for the substrate than the enzyme (Segel, 1993a). The data in the present study showed a similar trend (Table 2). Therefore, hakea may potentially inhibit the enzyme pyroglutamate aminopeptidase by forming a catalytically inactive pyroglutamate aminopeptidase-L-pyroglutamic acid β-napthylamide-hakea complex.

In order to confirm the type of inhibition of the enzyme, both Cornish-Bowden (data not shown) and Dixon (data not shown) plots were constructed. The two plots, in combination, provide an unambiguous indication of the type of inhibition (Cornish-Bowden, 1974). Various kinetic constants estimated from either the Cornish-Bowden plot or the Dixon plot are listed in Table 2.

The Hanes-Woolf plot (Fig. 2) as well as the Dixon and the Cornish-Bowden (data not shown) plots strongly indicated that the enzyme inhibition might follow mixed-type inhibition kinetics where the mixed-type system may be considered a combination of partial competitive and pure non-competitive inhibition (Segel, 1993a). Replots of the slopes and intercepts of the Hanes-Woolf plot were linear against the concentration of inhibitor used [I] ($r^2 = 0.972$ and 0.973, respectively) indicating that the inhibition was a linear mixed-type with $\alpha > 1$, $\beta = 0$ (intersecting, linear noncompetitive). The conclusion that ESI was catalytically inactive ($\beta = 0$) was supported by the linear Dixon plots ($r^2 = 0.981 - 0.995$ with different hakea concentrations) since Dixon plots for most mixed-type and partial inhibition systems are curved except when the ESI complex is catalytically inactive (Segel, 1993a). The value of αK_i was greater than K_i (Table 2) suggesting that binding of inhibitor to the ES form was stronger than the binding of inhibitor to the enzyme alone. The mean value of the variable α is greater for K_s than for K_i suggesting that the change in K_s is greater than the change in K_i (Table 2).

In conclusion, the natural oligosaccharide gum inhibits the enzyme pyroglutamate aminopeptidase and thus reduces the velocity of the reaction by forming a catalytically inactive enzyme-substrate-inhibitor complex. This natural gum may be a promising additive not only for its sustained-release and mucoadhesive properties (Alur et al., 1999, 1999a,b), but also for its ability to retard the enzymatic degradation of therapeutic polypeptides incorporated in dosage forms.

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