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Structure-activity relationships in the hydrolysis of acrylate and methacrylate esters by carboxylesterase in vitro

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Abstract

Acrylate esters are important chemicals in the plastics industry, whose toxicity is theorized to involve alkylation of critical cellular nucleophiles via the Michael addition. Carboxylesterase-mediated hydrolysis of acrylates may be a detoxification mechanism as the unsaturated acid produced is not electrophilic under physiological conditions. Using purified porcine liver carboxylesterase, the enzymatic hydrolysis of several acrylate esters was characterized to determine K_m and V_{max} values for each ester. The K_m (μM) and V_{max} (nmol/min) values observed for ethyl acrylate were 134 ± 16 (S.D.) and 8.9 ± 2.0, respectively. While the K_m for ethyl methacrylate was not significantly different, the V_{max}, 5.5 ± 2.5, was significantly lower compared with the corresponding value for ethyl acrylate. The K_m and V_{max} for butyl acrylate were 33.3 ± 8.5 μM and 1.49 ± 0.83 nmol/min, respectively, and the corresponding values for its α-methyl analog were not significantly different. The K_m and V_{max} for tetraethyleneglycol dimethacrylate were 39 ± 15 μM and 2.9 ± 1.0 nmol/min, respectively. The V_{max} for ethyleneglycol dimethacrylate, 6.9 ± 2.4 nmol/min, was significantly higher than that of the larger bifunctional ester tetraethyleneglycol dimethacrylate, but the K_m was not significantly different. These results indicate that α-methyl substitution appears to have a minor effect in the enzymatic hydrolysis of acrylates, and suggest that the relative toxicity of acrylates is not due to differences in carboxylesterase-mediated hydrolysis. Copyright © 1997 Elsevier Science Ireland Ltd.

Keywords: Acrylate esters; Methacrylate esters; Carboxylesterase; Structure-activity

1. Introduction

Acrylate and methacrylate esters are high volume chemicals used to make polymers for textiles, latex paints, surgical cements and dental resins.

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The unifying structural feature of this family of plastic monomers is the α,β-unsaturated ester functional group. The various esters differ by α-methyl substitution of the hydrogen (acrylates vs. methacrylates), alcohol/glycol moiety covalently bound to the carbonyl (e.g. methyl acrylate, ethyleneglycol dimethacrylate) and the number of α,β-unsaturated esters per molecule (mono-, bi-, trifunctional, etc.).

Acrylate esters are valuable monomers in the plastic industry, but they are not benign chemicals. They are known to cause dermal and sensory damage in exposed workers (Fries et al., 1975; Malten and Bende, 1979; Seppäläinen and Rajaniemi, 1984). Acrylates are direct-acting alkylating agents which readily deplete cellular thiols (Miller et al., 1981; Potter and Tran, 1992), an interaction which may be responsible for the toxicity of acrylates (Silver and Murphy, 1981; Frederick et al., 1990). The reactivity of acrylates towards glutathione (Tanii and Hashimoto, 1982; McCarthy et al., 1994) and their genotoxicity (Moore et al., 1988; Dearfield et al., 1989) are much greater than that of the corresponding methacrylates. In dermal carcinogenesis bioassays a few acrylates are weakly carcinogenic, yet to date no methacrylate ester tested has shown carcinogenic activity (DePass et al., 1984; Andrews and Clary, 1986; Wenzel-Hartung et al., 1989).

Acrylates have not shown carcinogenic activity when administered by inhalation, although nontumor lesions to olfactory mucosa were found. These included inflammation, hyperplasia, metaplasia and epithelial degeneration (Miller et al., 1985; Chan et al., 1988; Reininghaus et al., 1991). The activity of carboxylesterase, which hydrolyzes the ester to its unsaturated acid and alcohol, is much higher in the olfactory mucosa than in surrounding tissues (Olson et al., 1993). Non-tumor lesions in the nasal mucosa may be caused by localized irritation by the acrylate anion (Miller et al., 1985; Trela and Bogdanffy, 1991) which cannot alkylate critical cellular nucleophiles at physiological pH (Frederick and Reynolds, 1989).

The apparent V_{max} and K_m values for the carboxylesterase-mediated hydrolysis of methyl acrylate, ethyl acrylate and butyl acrylate were determined by Stott and McKenna (1985) using homogenates of mouse nasal mucosa. Enzymatic activity was lost in the millimolar acrylate concentration range, near the K_m values of the esters examined. The present research extends the studies by Stott and McKenna (1985) by using lower concentrations of acrylates, purified enzyme to limit ester loss to covalent binding, and a larger selection of esters including methacrylate esters (Fig. 1).

2. Materials and methods

2.1. Compounds

Ethyl acrylate (99% pure, stabilized with 15–20 ppm hydroquinone monomethyl ether, MEHQ, lot 07629CV), ethyl methacrylate (99% pure, 15 ppm MEHQ, lot 00211CV), butyl acrylate (99 + % pure, 10–55 ppm MEHQ, lot 02501 KM), tetraethyleneglycol diacrylate (95% pure, 225 ppm MEHQ, lot 03818AW), acrylic acid (99% pure, 200 ppm MEHQ, lot 04601LV) and methacrylic

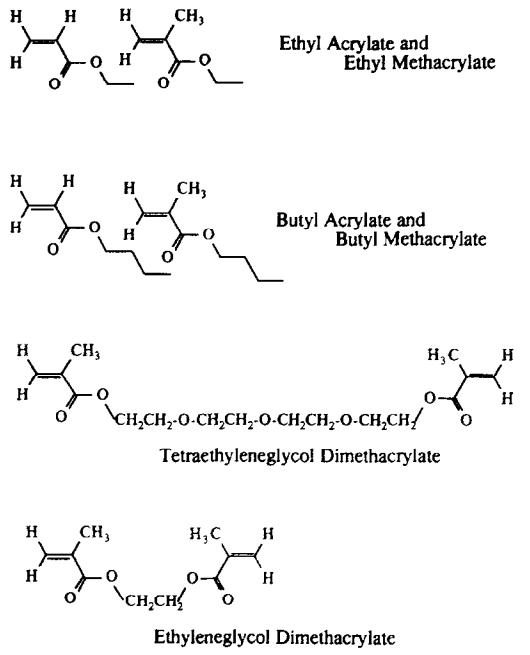

Fig. 1. Structures of acrylate- and methacrylate esters examined.

acid (98.5% pure, 250 ppm MEHQ, lot 06002HV) were purchased from Aldrich Chemical Co. (Milwaukee). Tetraethyleneglycol dimethacrylate (85% pure, 50–100 ppm HQ, lot GRL 1687), and ethyleneglycol dimethacrylate (85% pure, 40–150 ppm HQ, lot GSC 288) were gifts from Sartomer Company (West Chester, PA). Carboxylesterase (from porcine liver, lot 45F-8130) was purchased from Sigma (St. Louis) as a suspension in 3.2 M ammonium sulfate. Tris[hydroxymethyl]aminomethane (Tris, Sigma) was used as buffer (0.5 M, pH 8.0). 4-Nitrophenyl acetate (Aldrich) was used as substrate for enzyme standardization.

2.2. Carboxylesterase assay

Neat acrylate ester was initially diluted in ethanol followed by serial dilutions with 0.5 M Tris (pH 8.0) for final incubation concentrations of 0.005–2.5 μM ester in 1 ml. Carboxylesterase stock was diluted with Tris to a protein concentration of 10.7 μg/ml. Enzyme activity was standardized daily using 4-nitrophenyl acetate as substrate and measuring the absorbance at 405 nm of the chromophore 4-nitrophenol which is liberated upon enzymatic hydrolysis. The volume of the carboxylesterase stock solution (10.7 μg/ml) added to the acrylate solutions was adjusted for each experiment for standardization of enzymatic activity in the samples. About 25 μl of the carboxylesterase in Tris was added to 1 ml acrylate ester solution, in 3-ml capacity screw-capped vials and incubated for 20 min at 37°C in a shaking water bath. Preliminary experiments indicated that under these conditions hydrolysis was linear for more than 45 min. The reaction was terminated by acidification with 0.2 ml concentrated HCl. Methacrylic acid (5–500 μM final concentration) was added as an internal standard for acrylate ester incubations. Acrylic acid (5–500 μM final concentration) was added as an internal standard for methacrylate ester incubations. Acidified samples were extracted three times with ethyl acetate (1:1 v/v). The ethyl acetate fractions were pooled and concentrated under a stream of nitrogen gas. Acrylic acid and methacrylic acid was measured using a Varian 3700 gas chromatograph equipped with a flame ionization detector, a Supelco Nukol® wide bore capillary column (30 m $\times$ 0.53 mm i.d. $\times$ 0.5 μM film thickness) and a CDS 111 integrator. Injector, column and detector temperatures were 120, 90 and 140°C, respectively. Helium was the carrier gas with a flow rate of 20 ml/min through the column with an additional 10 ml/min through the detector. Hydrogen (30 ml/min) and air (300 ml/min) fueled the flame ionization detector. The gas chromatograph attenuator was set at 8, while its sensitivity was set at 10^{-11} amps/millivolt. The integrator attenuator was set at 16 with a chart speed of 0.25 cm/min.

2.3. Data analysis

The μmoles and mM concentration of acrylic acid or methacrylic acid generated in the incubation samples were calculated from the area under the peak of the acid produced, the area under the peak of fresh standard and the extraction efficiency of the internal standard. Apparent K_m and V_{max} values were determined from Eadie-Hofstee plots of the data and statistical analyses were performed using ANOVA. Experiments for each ester were performed three to seven times.

3. Results

Under the conditions described in Section 2, acrylic acid had a retention time of about 12 min and methacrylic acid had a retention time of about 16 min. The percent recovery of sample spiked with acrylic- and methacrylic acids ranged from 75 to 100%. The enzymatic constants K_m and V_{max} were determined for the hydrolysis of ethyl acrylate, ethyl methacrylate, butyl acrylate, butyl methacrylate, ethyleneglycol dimethacrylate and tetraethyleneglycol dimethacrylate mediated by porcine liver carboxylesterase in 0.5 M Tris buffer, pH 8.0. All acrylate esters examined were substrates for this enzyme (Table 1), with butyl acrylate exhibiting the lowest K_m (33 μM) and ethyl methacrylate exhibiting the highest K_m (159 μM). The lowest V_{max} was observed for butyl acrylate (1.49 nmol/min) while ethyl acrylate had the highest V_{max} (8.9 nmol/min). For ethyleneglyc-

Table 1
Hydrolysis of acrylate esters by porcine liver carboxylesterase in vitro

Ester	Concentation (μM)	n	K_m^a (μM)	V_{max}^a (nmol/min)	V_{max}/K_m (l/min)
Ethyl acrylate	25–2500	4	134 ± 16	8.9 ± 2.0	66
Ethyl methacrylate	25–2500	7	159 ± 90	5.2 ± 2.5*	33
Butyl acrylate	5–250	3	33.3 ± 8.5*	1.49 ± 0.83*	45
Butyl methacrylate	5–250	5	72 ± 28*	1.84 ± 0.64*	26
Ethyleneglycol dimethacrylate	5–250	4	64 ± 24*	6.9 ± 2.4†	108
Tetraethyleneglycol dimethacrylate	5–250	4	39 ± 15*	2.9 ± 1.0*‡	74

[a]Mean $\pm$ standard deviation.
*Significantly different at $P \leq 0.05$ compared with ethyl acrylate.
†Significantly different at $P \leq 0.05$ compared with butyl acrylate.
‡Significantly different at $P \leq 0.05$ compared with ethyleneglycol dimethacrylate.

col dimethacrylate, the moles of acid enzymatically produced was greater than moles of ester substrate at lower ester concentrations. This indicates that both ester functional groups are susceptible to enzymatic hydrolysis (data not shown).

α-Methyl substitution had only a minor effect upon K_m or V_{max}, indicating that acrylate esters are hydrolyzed to their non-electrophilic α,β-unsaturated acid as readily as their methacrylate ester analogs. The K_m value for ethyl methacrylate was not significantly different than that for ethyl acrylate, while the V_{max} value for the methacrylate was significantly lower. Neither K_m nor V_{max} values for butyl methacrylate were significantly different compared with the values for butyl acrylate.

The alcohol chain length significantly affected the K_m values for enzymatic hydrolysis of the monofunctional esters. Butyl acrylate had a K_m value four times lower compared with that for ethyl acrylate. The mean K_m value for butyl methacrylate was half that for ethyl methacrylate, but the standard deviations associated with both these mean values made this difference insignificant. The V_{max} for butyl acrylate was about six times slower than the V_{max} of ethyl acrylate, and the V_{max} of butyl methacrylate was almost three times slower than the V_{max} of ethyl methacrylate. For these monofunctional esters, longer alcohol chain length increases the affinity of the substrate for the active site of the enzyme, but turnover is slower. A comparison of the bifunctional esters ethyleneglycol dimethacrylate and tetraethyleneglycol dimethacrylate indicates no significant differences between their K_m values, however the V_{max} of ethyleneglycol dimethacrylate was significantly greater than that of tetraethyleneglycol dimethacrylate.

The determination of the enzymatic constants is performed experimentally at substrate saturating conditions. The ratio V_{max}/K_m is an indicator of a substrate's susceptibility to the enzymatic reaction at substrate sub-saturating conditions. The larger the ratio, the greater the susceptibility of the substrate to the enzymatic reaction. The V_{max}/K_m ratio was highest for ethyleneglycol dimethacrylate (108) and lowest for butyl methacrylate (26). In general the ratios were fairly similar among the various esters investigated (Table 1).

4. Discussion

Hydrolysis of acrylate esters results in the formation of acrylic acid and an alcohol. Acrylic acid, except under conditions of extremely low pH, is not electrophilic (Frederick and Reynolds, 1989). Therefore, hydrolysis may be an important detoxification pathway for acrylate esters. The present studies demonstrate that a variety of structurally different acrylates are substrates for carboxylesterase-mediated hydrolysis. They also show that α-methyl substitution does not significantly affect hydrolysis in comparison with the acrylate analog (Table 1).

Previous studies by Stott and McKenna (1985) reported K_m values of 3.14, 10.5 and 1.41 mM for the carboxylesterase-mediated hydrolysis of methyl-, ethyl- and butyl acrylate, respectively, in homogenates of mouse nasal mucosa. The apparent V_{max} for ethyl acrylate was estimated in these studies to be in the μM range. In the present studies, the K_m values for ethyl and butyl acrylate were found to be substantially lower, i.e. 133 and 33 μM, respectively, as were the V_{max} values which are in the nmol/min range (Table 1). These decreases in K_m and V_{max} values for acrylate hydrolysis are expected since purified carboxylesterase was used and non-esterase mediated reactions occurring in tissue homogenates did not take place.

Since the present studies were performed using porcine carboxylesterase, the question arises as to the relevance of the results to humans. Two isozymes from human liver were identified by isoelectric point, non-denaturing polyacrylamide gel electrophoresis, molecular weight, catalytic activity, N-terminus and immunological crossreactivity (Ketterman et al., 1989). The two human isozymes had similar catalytic properties to those of five purified rat liver carboxylesterases. Enzymatic and immunological properties of liver carboxylesterase purified from mouse, hamster, guinea pig, rabbit and monkey and partially purified from pig, cow, beagle and human were compared to three purified isozymes from rat (Hosokawa et al., 1990). Anti-rat RH1 cross-reacted with all isozymes tested except one isozyme from the monkey. When hydrolytic activity towards 4-nitrophenyl acetate and three other substrates was tested, the apparent K_m values for the hydrolysis of 4-nitrophenyl acetate for rat (RH1), rabbit (RB1) and monkey (MK1) were all similar to that reported for the enzymes from pig liver. No species tested demonstrated activity consistently similar to that of humans. A human liver carboxylesterase cDNA encoding about 80% of the enzyme had a high degree of similarity with the sequence of liver carboxylesterase from rat and rabbit (Riddles et al., 1991). As there is sequence and functional homology of carboxylesterase among mammalian species, porcine liver carboxylesterase can be used as a surrogate for the hydrolysis of esters in humans.

The present studies show that the V_{max}/K_m ratio, which, in this case, is an indicator of susceptibility to hydrolysis at sub-saturating substrate concentrations, is similar for the esters examined (Table 1). Acrylate toxicity appears to correlate with electrophilic reactivity, which is greatly diminished by the presence of an α-methyl substituent (Ghanayem et al., 1987; Frederick et al., 1990; McCarthy et al., 1994). The present results indicate that there appears to be no substantial difference in the susceptibility of hydrolysis with α-methyl substitution. The differences in tissue levels of carboxylesterase(s) may be important in tissue-specific toxicity of acrylates since metabolism by this enzyme is a detoxification pathway, but this pathway does not contribute to the structure-activity relationships for acrylate toxicity.

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