J. Chem. Thermodynamics 43 (2011) 235–239

Contents lists available at ScienceDirect

J. Chem. Thermodynamics

journal homepage: www.elsevier.com/locate/jct

A calorimetric and equilibrium investigation of the reaction {methyl ferulate(aq) + H_2O(l) = methanol(aq) + ferulic acid(aq)}

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ARTICLE INFO

Article history:
Received 18 August 2010
Accepted 1 September 2010
Available online 9 September 2010

Keywords:
Biomass
Calorimetry
Enthalpy
Entropy
Ferulic acid
Feruloyl esterase
Methyl ferulate
Methanol
Standard Gibbs free energy

ABSTRACT

Microcalorimetry and high-performance liquid chromatography (HPLC) have been used to conduct a thermodynamic investigation of the reaction: {methyl ferulate(aq) + H_2O(l) = methanol(aq) + ferulic acid(aq)}, as catalyzed by feruloyl esterase. Values of the apparent equilibrium constant $K' = (29.6 \pm 0.7)$ ($T = 298.15$ K, citrate buffer at pH 4.98, ionic strength $I = 0.39\ \mathrm{mol \cdot kg^{-1}}$) and of the calorimetrically determined enthalpy of reaction $\Delta_r H(\mathrm{cal}) = (4.0 \pm 0.9)\ \mathrm{kJ \cdot mol^{-1}}$ ($T = 298.15$ K and citrate buffer at pH 4.81, $I = 0.36\ \mathrm{mol \cdot kg^{-1}}$) were measured. A chemical equilibrium model, together with p*K*s and standard enthalpies of reaction $\Delta_r H^\circ$ for the H^+(aq) binding reactions of the reactants and products, was then used to calculate the values $K = (1.89 \pm 0.06) \cdot 10^{-4}$, $\Delta_r H^\circ = (7.3 \pm 1.7)\ \mathrm{kJ \cdot mol^{-1}}$, $\Delta_r G^\circ = (21.25 \pm 0.07)\ \mathrm{kJ \cdot mol^{-1}}$, and $\Delta_r S^\circ = -(46.8 \pm 5.7)\ \mathrm{J \cdot K^{-1} \cdot mol^{-1}}$ for the chemical reference reaction {methyl ferulate(aq) + H_2O(l) = methanol(aq) + ferulic acid$^-$(aq) + H^+(aq)}. These values of K and $\Delta_r H^\circ$ are similar in magnitude to the corresponding values reported for the reaction {propyl gallate(aq) + H_2O(l) = 3,4,5-trihydroxybenzoic acid$^-$(aq) + 1-propanol(aq) + H^+(aq)}. The results obtained in this study can be used in a chemical equilibrium model to calculate how K' and other standard transformed properties such as the standard transformed enthalpy $\Delta_r H'^\circ$, standard transformed Gibbs free energy $\Delta_r G'^\circ$, and the change in binding of H^+(aq), $\Delta_r N(H^+)$, vary with the independent variables T, pH, and I.

1. Introduction

Feruloyl esterase (EC 3.1.1.73) catalyses the hydrolysis of the feruloyl group (4-hydroxy-3-methoxycinnamoyl) from an esterified sugar, which is usually arabinose in natural substrates:

$$\begin{aligned}&\text{feruloyl-polysaccharide(aq)} + H_2O(l)\\ &= \text{ferulic acid(aq)} + \text{polysaccharide(aq)}. \qquad (1)\end{aligned}$$

This enzyme is classified as a hemicellulase accessory enzyme, since it helps xylanases and pectinases to break down the plant cell wall hemicellulose [1]. In the absence of a practically available substrate, feruloyl esterase is generally assayed by using methyl ferulate as the substrate in the biochemical reaction (see figure 1)

$$\text{methyl ferulate(aq)} + H_2O(l) = \text{methanol(aq)} + \text{ferulic acid(aq)}. \qquad (2)$$

Methyl ferulate also possesses some degree of structural similarity to feruloyl-polysaccharides, the natural substrates. Because efficient biomass utilization looks to breakdown acetylated xylans efficiently, knowledge of the thermodynamics of reactions that are catalyzed by feruloyl esterase assumes some practical importance. In this study, we measured the apparent equilibrium constant K' [2] for reaction (2) at $T = 298.15$, pH 4.98, and ionic strength $I = 0.39\ \mathrm{mol \cdot kg^{-1}}$. A calorimetrically determined enthalpy change $\Delta_r H(\mathrm{cal})$ for this reaction was also measured at $T = 298.15$, pH 4.81, and $I = 0.36\ \mathrm{mol \cdot kg^{-1}}$.

The results obtained in this study were treated by using a chemical equilibrium model [3,4] to obtain values of K and $\Delta_r H^\circ$ for a chemical reference reaction that involves specific ionic species rather than biochemical reactants that involve sums of species.

2. Experimental methods

2.1. Materials

Pertinent information on the substances used in this study is given in table 1.[1] The purities of those substances that are most

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[1] Certain commercial equipment, instruments, or materials are identified in this paper to specify the experimental procedures adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified is necessarily the best available for the purpose.

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doi:10.1016/j.jct.2010.09.002

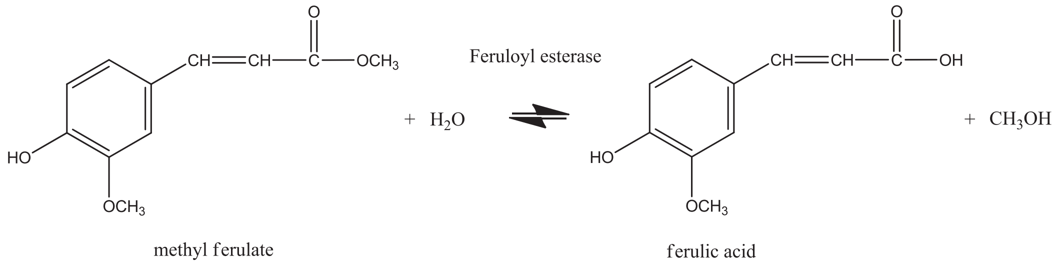

FIGURE 1. The structures of the substances and the reactions studied herein. The neutral forms of the aqueous species are shown.

TABLE 1
Principal substances used in this study with their Chemical Abstracts Service (CAS) registry numbers, empirical formulae, relative molecular masses M_r, mass fraction moisture contents w determined by Karl Fischer analysis, supplier (A = Alfa Aesar, I = IndoFine, L = LabChem, M = Mallinckrodt, S = Sigma–Aldrich), approximate mole fraction purities x, and the method(s) of analysis used by the vendors.

Substance[a,b]	CAS no.	Formula	M_r	w	Supplier	x[c]	Methods of analysis
Ferulic acid	1135-24-6	$C_{10}H_{10}O_4$	194.18	0.00020	I	0.998	HPLC
Feruloyl esterase[d]	134712-49-5		$3.4089 \cdot 10^4$		[d]		
Hydrochloric acid	7647-01-0	HCl	36.4606		L	>0.998	Titration
Methanol	67-56-1	CH_4O	32.0419	0.00058	S	>0.999	HPLC, fluorescence, titration
Methyl ferulate	2309-07-1	$C_{11}H_{12}O_4$	208.21	0.0044	A	0.997	GC
Sodium citrate tribasic dehydrate	6132-04-3	$C_6H_5Na_3O_7 \cdot 2H_2O$	294.10		M	>0.99	Trace metal analysis

[a] Except for sodium citrate tribasic dihydrate, the empirical formulas and the relative molecular masses refer to the anhydrous substances.
[b] Ferulic acid is also known as 4-hydroxy-3-methoxycinnamic acid and as 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid. Feruloyl esterase (EC 3.1.1.73) is also known as ferulic acid esterase and hydroxycinnamoyl esterase. Methyl ferulate is also known as 4-hydroxy-3-methoxycinnamic acid methyl ester and as 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid. Ferulic acid and ethyl ferulic acid are mixtures of their respective *cis* and *trans* forms.
[c] The estimated mole fraction purities are those reported by the vendor(s) and are exclusive of the amount of water in the samples. By using the chromatographic method described in Section 2.2, approximate mole fraction purities x were found to be 0.998 for ferulic acid, and 0.994 for methyl ferulate.
[d] Prepared for this study (see Section 2.1).

critical for this thermodynamic investigation were checked by using HPLC (see Section 2.2). Also, the mass fractions of water in these same substances were measured by performing Karl Fischer analyses with Metrohm 795 KFT Titrino and Metrohm 831 KF Titrino Coulometer automatic titrators. Hydranal Composite 2 and Hydranal Coulomat AG solutions, respectively, were used for the aforementioned instruments. Calibration of the Karl Fischer apparatuses was done by using a water-saturated octanol solution [5].

Feruloyl esterase A (EC 3.1.1.73) from *Penicillium funiculosum* was expressed in *Aspergillus awamori*. The gene was amplified by PCR from genomic *P. funiculosum* DNA, cloned into the pFE2 plasmid with its native signal sequence, and transformed into *A. awamori* through random chromosomal integration. The transformed host fungus was induced with maltose and the secreted enzyme was purified from the culture supernatant by ammonium sulfate precipitation followed by desalting, anion exchange, and size exclusion chromatography [6]. As prepared, the mass concentration of feruloyl esterase was $0.41\ \mathrm{mg \cdot cm^{-3}}$ in the buffer {$NaC_2H_3O_2$ (concentration $c = 0.020\ \mathrm{mol \cdot dm^{-3}}$) + NaCl ($c = 0.10\ \mathrm{mol \cdot dm^{-3}}$) + sodium azide ($c = 0.0031\ \mathrm{mol \cdot dm^{-3}}$), pH 5.0}. For use in the equilibrium and calorimetric experiments, this buffer was replaced with citrate buffer {$C_6H_5Na_3O_7$ (molality $m = 0.0917\ \mathrm{mol \cdot kg^{-1}}$) + HCl ($m = 0.0992\ \mathrm{mol \cdot kg^{-1}}$), pH 4.80}. This was accomplished either by dialysis (molar mass cut-off, MWCO = 3500) of the enzyme or by buffer exchange (Amicon Ultra 4 filter, MWCO = 5000). The enzyme was also concentrated by a factor of ≈10 by using an Amicon Ultra 4 filter, MWCO = 5000). The enzyme solution was kept frozen at $T = 253.15$ K and thawed just prior to use in the equilibrium and calorimetric experiments.

2.2. Chromatography

A Hewlett–Packard Model 1100 HPLC equipped with a UV detector set at the wavelength $\lambda = 320$ nm together with a Supelco Ascentis RP-Amide column no. 92165-01 (15 cm × 3 mm × 5 μm) and a C18 guard cartridge were used for the separation of methyl ferulate and ferulic acid. The column was thermostatted at $T = 303.15$ K. The mobile phases were (A) aqueous H_3PO_4 (mass fraction $w = 0.00085$) and (B) acetonitrile. The volume fractions ϕ of these mobile phases were: $\phi(\mathrm{A}) = 0.85$, $\phi(\mathrm{B}) = 0.15$ at time $t = 0$ and $\phi(\mathrm{A}) = 0.60$, $\phi(\mathrm{B}) = 0.40$ at $t = 30.0$ min. The flow rate was $0.0167\ \mathrm{cm^3 \cdot s^{-1}}$. Typical retention times were 7.7 min for ferulic acid and 15.7 min for methyl ferulate. The approximate limit of detection of methyl ferulate was $c = 3 \cdot 10^{-9}\ \mathrm{mol \cdot dm^{-3}}$.

2.3. Stock solution of methyl ferulate

The relatively low solubility of methyl ferulate combined with the slow rate at which it dissolved in the citrate buffer caused us to take an approach different than the direct weighing of that substance into the buffer. It was particularly important for the calorimetric measurements to use as high a concentration of methyl ferulate as possible given its relatively low solubility and, as later seen, the small molar enthalpy change for reaction (2). Thus, we prepared a solution of methyl ferulate in citrate buffer in which crystals of this substance were clearly visible. This solution was stirred and shaken for several hours in order to dissolve as much of the substance as possible. After allowing ≈1 h for the settling of the crystals, the majority of the clear supernatant solution was removed from the crystals by using a pipette. To be certain that no crystals remained and that the experiments were conducted safely below the saturation molality, additional citrate buffer was added to the clear supernatant solution so as to dilute the molality of the methyl ferulate by ≈20%. This final stock solution was used for the preparation of the solutions needed for both the equilibrium and the calorimetric measurements.

The molality of the methyl ferulate stock solution was measured by using the HPLC method described above (see Section 2.2). The measurement of the response factor of methyl ferulate was done by using gravimetrically prepared solutions of this substance in dimethyl sulfoxide, a solvent in which it was readily soluble. Note that, since the injection of samples into the HPLC was done by means of an injection loop having a fixed volume, it was necessary to make a correction for the fact that the density of the solution of methyl ferulate in aqueous citrate buffer is different than the density of the methyl ferulate in dimethyl sulfoxide. In performing this analysis, the area of the chromatographic peak corresponding to methyl ferulate in the response factor solution differed by only 0.08% from the area of the chromatographic peak that corresponded to methyl ferulate in the stock solution. The stock solution of methyl ferulate $\{m = (2.700 \pm 0.073) \cdot 10^{-3}\ \text{mol} \cdot \text{kg}^{-1}\}$ was kept at $T \approx 277$ K except when needed for the preparation of the solutions used in the equilibrium and calorimetric measurements. The aforementioned uncertainty in the value of m is based solely on the statistical uncertainties (two estimated standard deviations of the mean) in the values of the areas of the chromatographic peaks that correspond to methyl ferulate in the citrate buffer and in the DMSO solution that was used to establish the response factor for methyl ferulate. Additionally, based on chromatograms taken during the course of the series of experiments, there was no evidence for the decomposition of methyl ferulate in the stock solution. The decomposition of methyl ferulate at ambient temperature was studied directly and found to be negligible (<0.03% over a period of 72 h). This procedure permitted us to use a methyl ferulate stock solution that was close to the saturation molality, that was certain not to contain any un-dissolved crystals, and that had an accurately known molality.

2.4. Equilibrium measurements

The reaction mixture used for the forward direction of reaction (2) consisted of (methyl ferulate + methanol) in the citrate buffer. The reaction mixture used for the reverse direction of reaction consisted of (ferulic acid + methanol) in the citrate buffer. A substantial excess of methanol was used for the experiments involving both the forward and reverse directions of reaction. This was done in accordance with Le Châtelier's principle in order to have a sufficient amount of methyl ferulate in the reaction mixture to allow for an accurate chromatographic analysis. The forward and reverse reaction mixtures were allowed to proceed with gentle lateral shaking ($\approx$100 shakes $\cdot$ min^{-1}) in a thermostat set at $T = 298.15$ K. After an initial equilibration of 3 d and a second equilibration of 3 d, chromatographic analyses and calculation of the apparent reaction quotients Q' corresponding to the forward and reverse reaction mixtures showed that the reaction had not yet reached equilibrium. Thus, additional enzyme solution was added at each time to the forward and reverse reaction mixtures. Finally, after a total equilibration time of 10 d, the chromatographic analyses showed that the reaction had in fact reached equilibrium. All solutions used for the equilibrium and calorimetric experiments were prepared gravimetrically. Thus, the dilutions of the reaction mixtures caused by the addition of fresh enzyme solution could be accurately accounted for.

2.5. Calorimetric measurements

Descriptions of the microcalorimeters used in this study and their performance characteristics, the calibration and data-acquisition systems, and the computer programs used to treat the results have been given by Steckler *et al.* [7,8]. These calorimeters were calibrated electrically by using a high stability d.c. power supply, calibrated digital voltmeter, standard resistor, and time-interval counter. The electric potential differences U of the thermopiles in the microcalorimeters are measured with Agilent model 34420A Nanovolt Meters. The values of U are then recorded on a microcomputer and the areas of the thermograms are calculated by numerical integration. The calorimetric sample vessels were fabricated from high-density polyethylene. Each vessel had two compartments that held, respectively, $\approx$0.55 cm^3 and $\approx$0.40 cm^3 of solution. The substrate solution containing methyl ferulate was placed in the 0.55 cm^3 compartment and the enzyme solution containing feruloyl esterase was placed in the 0.40 cm^3 compartment. The substrate solution and the enzyme solution were prepared using the same citrate buffer. This helps to minimize any extraneous enthalpy changes due to a mismatch in the buffers.

The vessels and their contents were allowed to thermally equilibrate in the microcalorimeters for $\approx$60 min before the enzyme and substrate solutions were mixed. After mixing, $\approx$65 min was allowed for reaction. Following reaction, the vessels were removed from the microcalorimeters and the HPLC was used for the analysis of the final solutions. The fraction of unreacted methyl ferulate, which ranged from 0.001 to 0.01, was applied as a correction in the calculation of $\Delta_r H(\text{cal})$, the calorimetrically determined enthalpy of reaction (units of kJ $\cdot$ mol^{-1}) [2].

"Blank" enthalpy changes $\Delta_{\text{mix}}H$ were determined in control experiments. The average of the values of $\Delta_{\text{mix}}H$ for the mixing of the enzyme solution with the buffer was (0.89 ± 0.43) mJ and the average of the values of $\Delta_{\text{mix}}H$ for the mixing of the substrate solution with the buffer was (1.36 ± 0.43) mJ. The uncertainties given here are equal to two estimated standard deviations of the mean. We judge the total corrections applied for the blank enthalpy changes to be uncertain by ±0.61 mJ. The measured reaction enthalpies were $\approx$4 mJ. Thus, the uncertainties in the blank enthalpies correspond to an uncertainty of $0.15 \cdot \Delta_r H(\text{cal})$.

2.6. Measurement of pH

The pH measurements were done with a ThermoOrion Model 420 pH meter and a Radiometer combination glass micro-electrode at the temperature at which experiments were performed. The pH meter was calibrated with Radiometer standard buffers that bracketed the pHs of the solutions used in this study. The pHs of the reaction mixtures were calculated by using interpolation together with the measured electric potential differences and the pHs of the standard buffers.

3. Results and discussion

3.1. Thermodynamic formalism

The apparent equilibrium constant for reaction (2) is

$$K' = m(\text{methanol}) \cdot m(\text{ferulic acid})/\{m(\text{methyl ferulate}) \cdot m^\circ\}. \tag{3}$$

The molalities m in the above equations are the total molalities of the various ionic forms of the respective aqueous species. For example, $m(\text{ferulic acid}) = m(\text{ferulic acid}^0) + m(\text{ferulic acid}^-) + m(\text{ferulic acid}^{2-})$. The quantity m° ($m^\circ = 1$ mol $\cdot$ kg^{-1}) has been used to make K' dimensionless.

The reference reaction, which involves specific ionic species we have selected to correspond to the overall biochemical reaction (2), is:

$$\text{methyl ferulate(aq)} + \text{H}_2\text{O(l)} = \text{methanol(aq)} + \text{ferulic acid}^-(\text{aq}) + \text{H}^+(\text{aq}). \tag{4}$$

The equilibrium constant for this reaction is:

$$K = m(\text{methanol}) \cdot m(\text{ferulic acid}^-) \cdot m(\text{H}^+)/\{m(\text{methyl ferulate}) \cdot aw \cdot (m^\circ)^2\}, \tag{5}$$

The quantity a_w is the activity of water. In this study, the standard state for the solute is the hypothetical ideal solution of unit molality ($m^\circ = 1$ mol $\cdot$ kg^{-1}) and the standard state for the solvent is the pure solvent; the standard pressure $p^\circ = 0.1$ MPa.

It should be noted that the samples of methyl ferulate and ferulic acid used in this study were mixtures of their respective *cis* and *trans* isomers. Based solely on steric considerations, one would expect the *trans* form of these substances to be more stable than the *cis* form. Also, based on the structural similarity of methyl ferulate and ferulic acid, one would expect the respective fractions of the *cis* and *trans* isomers to be approximately the same for both substances. However, in the absence of knowledge of the equilibrium constants for the interconversion of the *cis* and *trans* isomers, we cannot calculate the actual fractions of these isomers in solution. Nevertheless, the absence of a detailed knowledge of the fractions of the *cis* and *trans* isomers does not prevent the formulation of the thermodynamics of reactions (2) and (4) as has been done above, where it is understood that one is dealing with a mixture of the respective *cis* and *trans* isomers of methyl ferulate and ferulic acid.

3.2. Results of experiments

The results of the equilibrium measurements are shown in table 2. We note the perfect and perhaps fortuitous agreement between the values of the apparent reaction quotient Q' obtained from both the forward and reverse directions of reaction. We take K' to be equal to the average of the values of Q'. We judge that reasonable estimates of possible systematic error in the values of K' are: $\pm 0.003 \cdot K'$ due to impurities (including water) in the samples; and $\pm 0.005 \cdot K'$ due to possible errors in the chromatographic analyses. These estimates of possible systematic error are combined in quadrature together with the statistical uncertainty in the measured value of K', expressed as one estimated standard deviation of the mean, to obtain a combined standard uncertainty [9]. This combined standard uncertainty is then multiplied by two to arrive at the final result ($T = 298.15$ K and citrate buffer at pH 4.98): $K' = (29.6 \pm 0.7)$ for reaction (2).

The results of the calorimetric measurements are given in table 3. We judge that reasonable estimates of possible systematic error in the values of $\Delta_r H(\text{cal})$ are: $\pm 0.003 \cdot \Delta_r H(\text{cal})$ due to impurities (including water) in the methyl ferulate sample; $\pm 0.001 \cdot \Delta_r H(\text{cal})$ due to possible errors in the extents of reaction; $\pm 0.014 \cdot \Delta_r H(\text{cal})$ due to possible errors in the measurement of the concentration of the methyl ferulate stock solution (see Section 2.3); and $\pm 0.08 \cdot \Delta_r H(\text{cal})$ due to possible errors in the calorimetric measurements, almost all of which is due to the uncertainty in the "blank" enthalpies. These estimates of possible systematic error are combined in quadrature together with the statistical uncertainty in the measured value of $\Delta_r H(\text{cal})$, expressed as one estimated standard deviation of the mean, to obtain a combined standard uncertainty [9]. This combined standard uncertainty is then multiplied by two to arrive at the final result ($T = 298.15$ K and citrate buffer at pH 4.81 and $I = 0.36\ \text{mol} \cdot \text{kg}^{-1}$): $\Delta_r H(\text{cal}) = (4.0 \pm 0.9)\ \text{kJ} \cdot \text{mol}^{-1}$ for reaction (2).

3.3. Equilibrium modeling calculations

The pK values and standard enthalpy changes $\Delta_r H^\circ$ for the proton dissociation reactions of the reactants and of the buffer are needed to relate the experimental results obtained in this study to standard thermodynamic quantities for the reference reactions (see Section 3.1). These pK and $\Delta_r H^\circ$ values are given in table 4 together with the basis for these values. In a few cases, it was necessary to adjust pK and $\Delta_r H^\circ$ values to $I = 0$. This was done by using the extended Debye–Hückel equation with the "ion-size" parameter set at $1.6\ \text{kg}^{1/2} \cdot \text{mol}^{-1/2}$ [3]. The equilibrium model used for the calculation of the equilibrium constants K and standard enthalpies $\Delta_r H^\circ$ for the reference reactions from the measured values of K' and $\Delta_r H(\text{cal})$ has been described [3,4]. The calculations include corrections for non-ideality and are made self-consistent in regard to the ionic strength. The non-ideality corrections are based on the extended Debye–Hückel equation in which the "ion-size" parameter has been set at $1.6\ \text{kg}^{1/2} \cdot \text{mol}^{-1/2}$. Use of the equilibrium model

TABLE 4

The pKs and standard enthalpy changes $\Delta_r H^\circ$; at $T = 298.15$ K and at the ionic strength $I = 0$ for the aqueous H^+ dissociation reactions of substances pertinent to this study.[a,b]

Reaction		pK	$\Delta_r H^\circ/(\text{kJ} \cdot \text{mol}^{-1})$
$H_3\text{citrate}^0 = H^+ + H_2\text{citrate}^-$	(6)	3.128[c]	4.07[c]
$H_2\text{citrate}^- = H^+ + H\text{citrate}^{2-}$	(7)	4.761[c]	2.23[c]
$H\text{citrate}^{2-} = H^+ + \text{citrate}^{3-}$	(8)	6.396[c]	−3.38[c]
$\text{Ferulic acid}^0 = H^+ + \text{ferulic acid}^-$	(9)	4.29[d]	4.1[d]
$\text{Ferulic acid}^- = H^+ + \text{ferulic acid}^{2-}$	(10)	8.75[d]	19.0[d]
$\text{Methyl ferulate}^0 = H^+ + \text{methyl ferulate}^-$	(11)	8.75[e]	19.0[e]

[a] The standard state for the solutes is the hypothetical ideal solution of unit molality. Citrate^{3-} is $C_6H_5O_7^{3-}$, ferulic acid^{2-} is $C_{10}H_8O_4^{2-}$, and methyl ferulate$^-$ is $C_{11}H_{11}O_4^-$.

[b] For the purpose of assessing possible errors attributable to the equilibrium model (see Section 3.3), the values of pK and $\Delta_r H^\circ/(\text{kJ} \cdot \text{mol}^{-1})$ are, respectively, estimated to be uncertain by the following: reaction (6), ±0.003 and ±0.2; reaction (7), ±0.003 and ±0.2; reaction (8), ±0.003 and ±0.2; reaction (9), ±0.05 and ± 1.0; reaction (10), ±0.3 and ±1.0 and reaction (11), ±0.3 and ±2.0.

[c] Based on the selected pK and $\Delta_r H^\circ$ values given by Goldberg *et al.* [10].

[d] Nordström and Lindberg [11] report p$K = 4.52$ for reaction (9) and p$K = 9.39$ for reaction (10) at $T = 298.15$ K and $I = 0.1\ \text{mol} \cdot \text{dm}^{-3}$. Adjustment of these values (see Section 3.3) gives p$K = 4.32$ for reaction (9) and p$K = 8.94$ at $I = 0$ for reaction (10) at $I = 0$. Shtreis and Nikitin [12] report p$K = 9.47$ for reaction (10), but did not report a temperature or ionic strength. Casolaro *et al.* [13] report p$K = 4.48$ for reaction (9) and p$K = 8.94$ for reaction (10) at $T = 298.15$ K and $I = 0.15\ \text{mol} \cdot \text{dm}^{-3}$. Adjustment of these values gives p$K = 4.26$ for reaction (9) and p$K = 8.49$ for reaction (10) at $I = 0$. Fazary and Ju [14] report p$K = 4.50$ for reaction (9) and p$K = 9.21$ for reaction (10) at $T = 298.15$ K and $I = 0.1\ \text{mol} \cdot \text{dm}^{-3}$. Adjustment of these values gives p$K = 4.30$ for reaction (9) and p$K = 8.81$ for reaction (10) at $I = 0$. Mota *et al.* [15] report p$K = 4.61$ for reaction (9) at $T = 298.15$ K but do not give the ionic strength. The selected pK values are the averages of the pK values at $I = 0$ obtained from the studies of Nordström and Lindberg [11], Casolaro *et al.* [13], and Fazary and Ju [14]. The selected values of $\Delta_r H^\circ$ are based on the results of Casolaro *et al.* [13].

[e] Estimated value based on the structural similarity of ferulic acid and methyl ferulate.

TABLE 2

Results of equilibrium measurements at $T = 298.15$ K and pH 4.98 for the reaction {methyl ferulate(aq) = ferulic acid(aq) + methanol(aq)}[a].

Experiment	$m(\text{citrate})/(\text{mol} \cdot \text{kg}^{-1})$	$m(\text{HCl})/(\text{mol} \cdot \text{kg}^{-1})$	$m(\text{methyl ferulate})/(\text{mol} \cdot \text{kg}^{-1})$	$m(\text{methanol})/(\text{mol} \cdot \text{kg}^{-1})$	$m(\text{ferulic acid})/(\text{mol} \cdot \text{kg}^{-1})$	$I/(\text{mol} \cdot \text{kg}^{-1})$	Q'
Forward	0.0895	0.0968	0.000135	3.17	0.00126	0.39	29.6 ± 0.6
Reverse	0.0907	0.0980	0.000482	3.63	0.00393	0.39	29.6 ± 0.6
			$K' = 29.6 \pm 0.6$				

[a] The molalities m of the citrate buffer components and of the reactants at equilibrium are given in columns 2 to 6. The approximate mass fraction of the feruloyl esterase was 0.0018 in the forward reaction and 0.0009 in the reverse reaction. The value of the ionic strength I is calculated. The uncertainties in the values of the apparent reaction quotient Q' are based on the statistical uncertainties (two estimated standard deviations of the mean) in the areas of the chromatographic peaks (including the determination of the response factor) used to establish the concentration of methyl ferulate in the reaction mixtures. K' is the apparent equilibrium constant.

TABLE 3

Results of calorimetric measurements at $T = 298.15$ K and pH 4.81 for the reaction {methyl ferulate(aq) = ferulic acid(aq) + methanol(aq)}.[a]

Experiment no.	$m(\text{citrate})/(\text{mol} \cdot \text{kg}^{-1})$	$m(\text{HCl})/(\text{mol} \cdot \text{kg}^{-1})$	$m(\text{methyl ferulate})/(\text{mol} \cdot \text{kg}^{-1})$	$I/(\text{mol} \cdot \text{kg}^{-1})$	$\Delta_r H(\text{cal})/(\text{kJ} \cdot \text{mol}^{-1})$
1	0.0894	0.0967	0.001602	0.36	4.62
2	0.0894	0.0967	0.001747	0.36	3.36
3	0.0894	0.0967	0.001851	0.36	4.01
4	0.0894	0.0967	0.001787	0.36	5.05
5	0.0894	0.0967	0.001669	0.36	3.68
6	0.0894	0.0967	0.001675	0.36	3.27
			$\langle \Delta_r H(\text{cal}) \rangle = (4.00 \pm 0.58)\ \text{kJ} \cdot \text{mol}^{-1}$		

[a] The molalities m of the citrate buffer components and of the methyl ferulate are given in columns 2 to 4. These molalities are those obtained after mixing of the enzyme and substrate solutions and prior to any reaction. The approximate mass fraction of the feruloyl esterase was 0.0015. The value of the ionic strength I is calculated. The uncertainty in the average value of the calorimetrically determined enthalpy change $\Delta_r H(\text{cal})$ is equal to two estimated standard deviations of the mean.

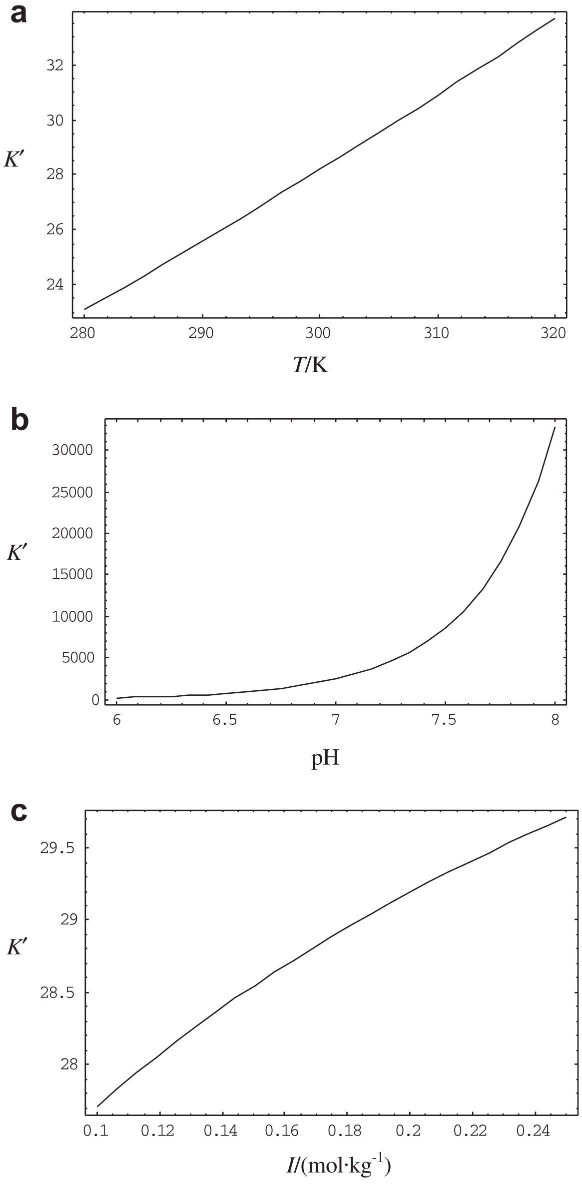

FIGURE 2. Plots of the apparent equilibrium constant K' as functions of temperature, pH, and ionic strength: (a) pH 5.0 and $I = 0.10\ \mathrm{mol \cdot kg^{-1}}$; (b) $T = 298.15$ K and $I = 0.10\ \mathrm{mol \cdot kg^{-1}}$; and (c) pH 5.0 and $T = 298.15$ K.

with the experimental results for K' and $\Delta_r H(\mathrm{cal})$ and with the thermodynamic quantities given in table 4, leads to the values $K = (1.89 \pm 0.05) \cdot 10^{-4}$ and $\Delta_r H^\circ = (7.3 \pm 1.7)\ \mathrm{kJ \cdot mol^{-1}}$ for the chemical reference reaction (4) at $T = 298.15$ K and ionic strength $I = 0$. The calculated value of $\Delta_r N(\mathrm{H^+})$, the change in binding of $\mathrm{H^+(aq)}$ accompanying the overall biochemical reaction (2) under the conditions (T, pH, and I) the experiments were performed in the calorimetric measurements, is -0.826. This calculated value was used in performing the buffer protonation correction [16]. The equilibrium model was also used to calculate the ionic strength values. The calculated values of K and $\Delta_r H^\circ$ lead to $\Delta_r G^\circ = (21.25 \pm 0.07)\ \mathrm{kJ \cdot mol^{-1}}$ and $\Delta_r S^\circ = -(46.8 \pm 5.7)\ \mathrm{J \cdot K^{-1} \cdot mol^{-1}}$ for the chemical reference reaction (4) at $T = 298.15$ K and $I = 0$.

The uncertainties discussed thus far are based on the uncertainties in the experimentally determined values of K' and $\Delta_r H(\mathrm{cal})$. However, there is also a component of uncertainty due to uncertainties in the parameters used in the equilibrium model. This latter component of uncertainty was examined by perturbing each of the pertinent quantities in the model. These perturbations are summarized in table 4 (see footnote "b"). The "ion-size" parameter used in the activity coefficient model was also perturbed by $\pm 0.3\ \mathrm{kg^{1/2} \cdot mol^{-1/2}}$. Thus, the final results are $K = (1.89 \pm 0.06) \cdot 10^{-4}$, $\Delta_r H^\circ = (7.3 \pm 1.7)\ \mathrm{kJ \cdot mol^{-1}}$, $\Delta_r G^\circ = (21.25 \pm 0.08)\ \mathrm{kJ \cdot mol^{-1}}$, and $\Delta_r S^\circ = -(46.8 \pm 5.7)\ \mathrm{J \cdot K^{-1} \cdot mol^{-1}}$ for the chemical reference reaction (4) at $T = 298.15$ K and $I = 0$.

3.4. Comparisons with other results

This study is the first to report values of K' and $\Delta_r H(\mathrm{cal})$ for reaction (2) [17]. Indeed, the thermochemical literature on ferulic acid and methyl ferulate is sparse. In addition to the pK and $\Delta_r H^\circ$ vales given in table 4, the only other thermochemical data in the literature is that of Mota *et al.* [15], who report the melting temperature, the molar enthalpy of fusion, and the solubility of ferulic acid in water from $T = (288\ \text{to}\ 323)$ K. At $T = 298.15$ K, the solubility of ferulic acid is $0.78\ \mathrm{g \cdot dm^{-3}}$ [15]. This corresponds to a saturation molality $m(\mathrm{sat}) = 0.00403\ \mathrm{mol \cdot kg^{-1}}$. In any case, in the absence of the thermochemical data, it is not possible to use thermochemical pathways to calculate values of K and $\Delta_r H^\circ$ for reaction (4) that would permit a comparison with the results of this study. However, a somewhat structurally similar reaction is

$$\text{3,4,5-trihydroxybenzoic acid propyl ester(aq)} + \mathrm{H_2O(l)} = \text{3,4,5-trihydroxybenzoic acid(aq)} + \text{1-propanol(aq)}, \quad (12)$$

Tewari *et al.* [18] measured values of K' and $\Delta_r H(\mathrm{cal})$ for reaction (12) and then used an equilibrium model to calculate $K = 4.37 \cdot 10^{-4}$ and $\Delta_r H^\circ = -4.6\ \mathrm{kJ \cdot mol^{-1}}$ for the chemical reference reaction

$$\text{3,4,5-trihydroxybenzoic acid propyl ester(aq)} + \mathrm{H_2O(l)} = \text{3,4,5-trihydroxybenzoic acid}^{-}\text{(aq)} + \text{1-propanol(aq)} + \mathrm{H^+(aq)}. \quad (13)$$

These values are similar in magnitude to the values $K = (1.89 \pm 0.06) \cdot 10^{-4}$ and $\Delta_r H^\circ = (7.3 \pm 1.7)\ \mathrm{kJ \cdot mol^{-1}}$ obtained in this study for reaction (4).

3.5. Predictive modeling calculations

The results obtained in this study, together with the equilibrium model [4], can be used to calculate how K' and other standard transformed properties [2] such as $\Delta_r H'^\circ$, $\Delta_r G'^\circ$, and $\Delta_r N(\mathrm{H^+})$, vary with the independent variables T, pH, and I. As examples, plots of K' as a function of temperature, pH, and ionic strength are shown in figure 2. It is seen that there is a very substantial increase in the value of K' with increasing pH.

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