in which it was absent (G-8, H-15) or very low, as in the turbinado sugars, are not perceived by tasters as normal brown sugars.

ACKNOWLEDGMENT

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Registry No. Acetic acid, 64-19-7; sucrose, 57-50-1; acetaldehyde, 75-07-0; diacetyl, 431-03-8; methanol, 67-56-1; ethanol, 64-17-5.

LITERATURE CITED

Flink, J.; Karel, M. *J. Agric. Food Chem.* **1970**, *18*, 295–297.
Godshall, M. A.; Legendre, M. G.; Roberts, E. J. *J. Agric. Food Chem.* **1980**, *28*, 856–858.
Godshall, M. A.; Roberts, E. J. *Proc. Tech. Sess. Cane Sugar Refin. Res.* **1980**, 26.
Legendre, M. G.; Fisher, G. S.; Dupuy, H. P.; Rayner, E. T. *J. Am. Oil Chem. Soc.* **1979**, *56*, 552.
Niediek, E. A.; Babernics, L. *Gordian* **1979**, *79*, 35–36, 38–40, 42–44.
Massaldi, H. A.; King, C. J. *J. Food Sci.* **1974**, *39*, 438–444.
Reineccius, G. A.; Coulter, S. T. *J. Dairy Sci.* **1969**, *52*, 1219–1223.
Trombella, B.; Ribeiro, A. *Am. J. Enol. Vitic.* **1980**, *31*, 294–297.

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Kinetics of Hydrolysis of Dimeric and Trimeric Methionine Hydroxy Analogue Free Acid under Physiological Conditions of pH and Temperature

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Liquid feed supplements based on DL-methionine hydroxy analogue free acid (DL-MHA free acid, DL-2-hydroxy-4-(methylthio)butyric acid) contain considerable amounts of oligomeric MHA free acids. As a first approach to physiological decomposition the hydrolysis of dimeric and trimeric MHA free acid in dilute hydrochloric acid solution is investigated. By use of HPLC it is possible to follow the changes in concentration of all species. The hydrolysis reactions are found to be first order with respect to the oligomer concentration. The rate constant increases with HCl concentration and temperature (activation energy for dimeric MHA free acid: 72 kJ/mol). The rate constants obtained at 37 °C in 0.1 N aqueous HCl correspond to half-lives of 1.8 and 1.6 days for dimeric and trimeric MHA free acid, respectively. The results indicate that oligomeric MHA free acids show rather slow hydrolysis under physiological conditions of pH and temperature.

Essential amino acids such as DL-methionine are of general importance in the animal nutrition industry as supplements to broiler feed, hog feed, etc., in order to increase the protein value. Crystalline DL-methionine, purified to a minimum of 99% by weight, is the major commercial product used. A substitute product, which is used to a lesser extent, is the calcium salt of DL-2-hydroxy-4-(methylthio)butyric acid (MHA, a registered trademark of Monsanto Co.) with a purity of 93% by weight.

A liquid product containing 88% of DL-MHA free acid (DL-methionine hydroxy analogue free acid, DL-2-hydroxy-4-(methylthio)butyric acid) (Alimet, a registered trademark of Monsanto Co.) has recently been introduced as a feed supplement. Like other α-hydroxycarboxylic acids, DL-MHA free acid inevitably polymerizes in concentrated solution. This polymerization occurs via intermolecular esterification and results in a mixture of different stereoisomers. It was reported that an equilibrium solution of 85% DL-MHA free acid contains 64.5% by weight monomeric DL-MHA free acid, 20.1% by weight dimeric (linear and cyclic) MHA free acid, and 1.3% by weight trimeric MHA free acid (Cummins, 1973). A recent investigation on 88% DL-MHA free acid solution (Ivey, 1981) indicated that the equilibrium concentrations of polymers are even higher (17.6% by weight dimeric MHA free acid, 8.8% by weight trimeric MHA free acid, and 4.4% by weight higher oligomers). These oligomers are significant because it has been reported that the polymeric MHA free acids "are not useful as feed components" (Nufer, 1966). Boebel and Baker (1982) found that MHA free acid polymers had an efficacy only 54% that of DL-methionine when compared on a molar basis.

So with regard to the nutritional value one must assume that a hydrolysis of the polymers to the monomer is needed. As a first approach to physiological decomposition we have investigated the hydrolysis of dimeric and trimeric MHA free acid in dilute hydrochloric acid solution as a function of HCl concentration and temperature.

MATERIALS AND METHODS

Solutions were concentrated in a rotary evaporator. The existence of mono-, di- and trimeric MHA free acid was verified by ^{1}H NMR, IR, and mass spectra.

Monomeric DL-MHA free acid (I) was prepared by the following preparative route via ester saponification: DL-MHA-Ca (548 g) was dissolved in water (4.5 L) and activated charcoal and kieselgur were added. The mixture was then heated and filtered while still hot. To remove Ca, the filtrate was passed through an ion-exchange column (Lewatit S 100 from Bayer, Federal Republic of Germany). Concentrating the eluate gave DL-MHA free acid (451 g,

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92.7%). To form the ester, DL-MHA free acid (736 g) was refluxed with methanol (2.8 L) for 3 h in the presence of Lewatit S 100 (120 g) as a catalyst.

After evaporation of the excess methanol, the esterification process was repeated. The resulting crude DL-MHA methyl ester (771.9 g, 95.9%) was distilled (bp 79–81 °C at 0.13 mbar).

The following ester saponification gave a very dilute aqueous solution of the monomer, so that polymerization was minimized. By concentrating this solution at low temperature, we obtained the pure monomer.

DL-MHA methyl ester (70 g) was stirred in 1 N NaOH (460 mL) for 5 h at room temperature. To remove Na, the reaction solution was passed through an ion-exchange column (Lewatit S 100). Concentrating the eluate at 50 °C gave monomeric DL-MHA free acid as a light yellow solid melting at room temperature. The HPLC chromatogram showed a large peak for monomeric DL-MHA free acid and none for dimeric MHA free acid or higher oligomers. The purity of the product, determined by titration with aqueous NaOH, was 96% by weight. The residue was 3% by weight water (by Karl Fischer) and 1% by weight DL-MHA methyl ester (by HPLC).

Dimeric MHA Free Acid (II). Monomeric DL-MHA free acid was heated in a sealed glass ampule for 100 h at 70 °C. Under these conditions part of the monomer polymerized to dimeric and trimeric MHA free acid. Preparative HPLC afforded a fraction containing the dimeric MHA free acid. This fraction was neutralized with 0.1 N NaOH, concentrated at 40 °C, adjusted to pH 2 with H_3PO_4, and extracted by using CH_2Cl_2. Concentrating the extract at 40 °C gave dimeric MHA free acid as a chromatographically pure pale pink syrup. Complete hydrolysis of this product to monomeric DL-MHA free acid (conditions: 0.2 N aqueous HCl; 37 °C; 193 h), gave a purity of 98.2% by weight. The preparative chromatographic separation was performed on a 250 × 16 mm i.d. column packed with 10-μm LiChrosorb RP-18 (Knauer, Federal Republic of Germany) with a flow rate of 18 mL/min. The eluent was a mixture (v/v) of 30% acetonitrile and 70% 0.005 M aqueous phosphoric acid. The UV monitor was set at 205 nm.

Trimeric MHA free acid (III) was isolated by preparative HPLC under conditions analogous to those described above. In order to produce a higher percentage of trimer, the monomeric DL-MHA free acid was heated for more than 100 h. Diethyl ether was used for the extraction instead of CH_2Cl_2. The trimeric MHA free acid was obtained as a light brown syrup. Complete hydrolysis to monomeric DL-MHA free acid (conditions: 0.1 N aqueous HCl; 37 °C; 503 h) gave a purity of 96.6% by weight. The preparative chromatographic separation was performed with an eluent of 50% acetonitrile and 50% 0.005 M aqueous phosphoric acid (v/v) and a flow rate of 30 mL/min.

Kinetic Measurements. Aqueous HCl (Merck, Federal Republic of Germany) was added to a weighed amount of dimeric and trimeric MHA free acid, respectively, in a 15-mL glass ampule. The ampule was covered with a septum and put into a constant-temperature bath (time t = 0). Samples (5 μL) were taken with a syringe after various time intervals. The change in concentration of dimeric and trimeric MHA free acid, respectively, and monomeric DL-MHA free acid was followed by analytical HPLC.

Analytical HPLC. The high-pressure liquid chromatography (HPLC) system was composed of a Model 600/200 pump (Gynkotek, Federal Republic of Germany), a Rheodyne Model 7105 sample injector, an ultraviolet detector (Uvidec-100-III from Jasco, Japan), set at a wavelength of 205 nm, and an integrator (SP 4100, Spectra-Physics). The 250 × 4.6 mm i.d. stainless steel column was packed with 7-μm LiChrosorb Si 60 (Merck, Federal Republic of Germany), which was silanized with Si 118 (Degussa, Inc.).

The eluent was a mixture (v/v) of 50% acetonitrile and 50% 0.005 M aqueous phosphoric acid (flow rate 2 mL/min). The samples (5 μL) taken from the kinetic experiments were injected directly. Standard solutions of mono-, di- and trimeric MHA free acid were used for the calibration.

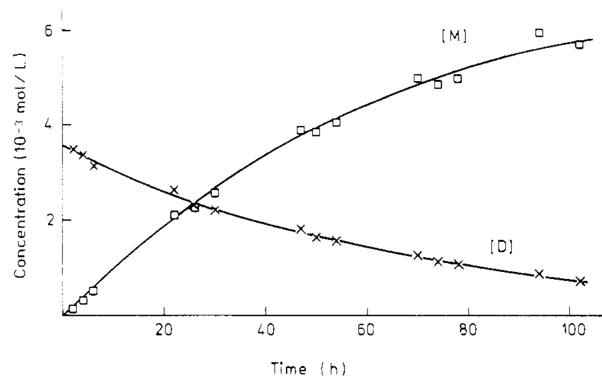


Figure 1. Hydrolysis of dimeric MHA free acid (initial concentration 1 g/L) in 0.1 N aqueous HCl at 37 °C. ([M] and [D] = concentrations of mono- and dimeric MHA free acid, respectively.)

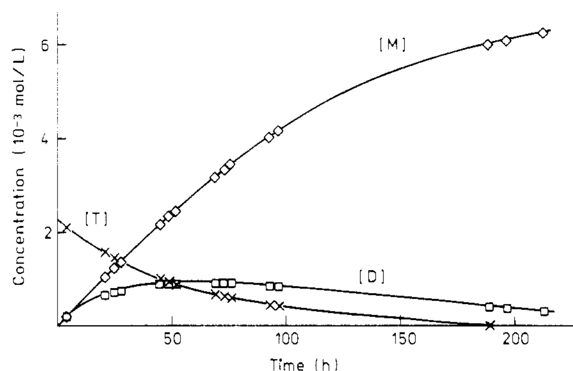
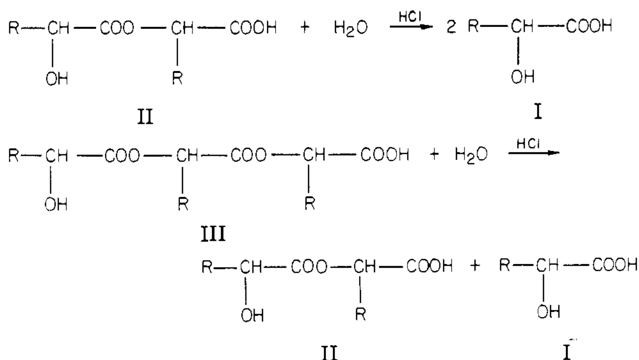


Figure 2. Hydrolysis of trimeric MHA free acid (initial concentration 1 g/L) in 0.1 N aqueous HCl at 37 °C. ([M], [D], and [T] = concentrations of mono-, di-, and trimeric MHA free acid, respectively.)

RESULTS AND DISCUSSION

Reactions. Monomeric DL-MHA free acid (I) is the final product of the acid hydrolysis of dimeric (II) and trimeric (III) MHA free acid (R = CH_3-S-CH_2-CH_2):



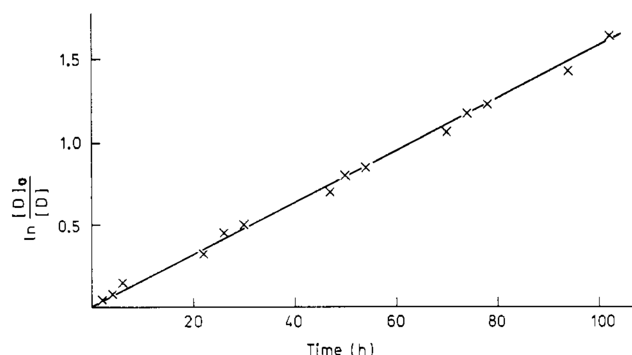


Figure 3. Evaluation of rate constant for the hydrolysis of dimeric MHA free acid in 0.1 N aqueous HCl at 37 °C. ([D] = concentration of dimeric MHA free acid; subscript 0 indicates initial concentration.)

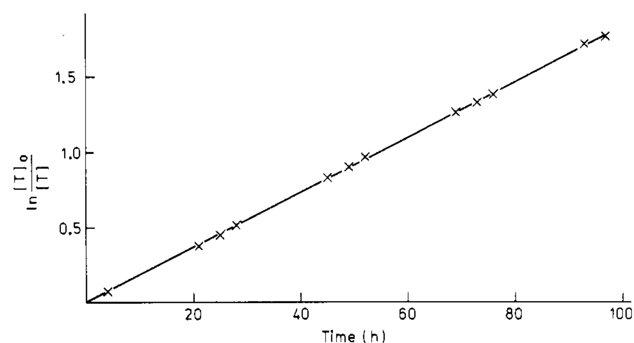


Figure 4. Evaluation of rate constant for the hydrolysis of trimeric MHA free acid in 0.1 N aqueous HCl at 37 °C. ([T] = concentration of trimeric MHA free acid; subscript 0 indicates initial concentration.)

Table I. Rate Constants for the Hydrolysis of Di- and Trimeric MHA Free Acid in 0.1 N Aqueous HCl at 37 °C

initial concn, g/L	k_D, 10^{-2} h^{-1}	k_T, 10^{-2} h^{-1}
0.5	1.69	1.85
1	1.57	1.85, 1.84[a]

[a] k_T at a concentration of 1 g/L was measured twice.

Table II. Rate Constants for the Hydrolysis of Dimeric MHA Free Acid (Initial Concentration 1 g/L) in Various Concentrations of Aqueous HCl at 37 °C

[HCl], mol/L	0.05	0.1	0.216
k_D, 10^{-2} h^{-1}	0.754	1.57	3.3

Using HPLC it is possible to evaluate the changes in concentration of all species. Typical concentration/time data are shown in Figures 1 and 2.

Rate Laws and Rate Constants. The hydrolysis reactions of dimeric and trimeric MHA free acid were found to follow first-order rate laws

$$d[D]/dt = -k_D[D] \quad (1)$$

$$d[T]/dt = -k_T[T] \quad (2)$$

where [D] and [T] are the concentrations of the dimeric and trimeric MHA free acid, respectively, in mol/L, k_D and k_T are the rate constants in h^{-1}, and t is the reaction time in h.

By use of the integrated equations

$$\ln([D]_0/[D]) = k_D t \quad (3)$$

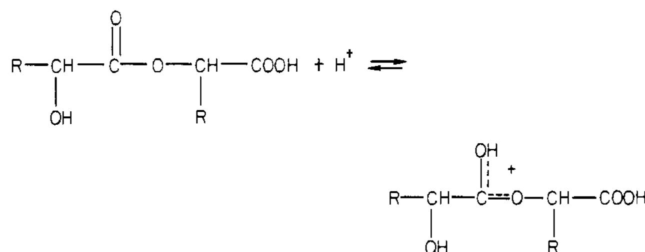
$$\ln([T]_0/[T]) = k_T t \quad (4)$$

where the subscript 0 indicates initial concentrations, rate constants were evaluated by plotting ln ($[D]_0/[D]$) and ln ($[T]_0/[T]$) against t. Figures 3 and 4 show one example of each case. Rate constants obtained at two initial concentrations of dimeric and trimeric MHA free acid are summarized in Table I. Within experimental error the rate constants are independent of the initial concentrations.

Influence of HCl Concentration and Temperature. The influence of HCl concentration and temperature was studied for dimeric MHA free acid only. From the data in Table II it can be seen that the rate constant increases linearly with HCl concentration. This result is to be expected from theoretical considerations since the mechanisms of acid-catalyzed ester hydrolysis (Ingold, 1969) involve preequilibrium with a proton adding according to

Table III. Rate Constants for the Hydrolysis of Dimeric MHA Free Acid (Initial Concentration 1 g/L) in 0.1 N Aqueous HCl at Various Temperatures

temperature, °C	25	37	50
k_D, 10^{-2} h^{-1}	0.466	1.57	4.51



followed by the rate-determining step.

The temperature dependence is shown in Table III. By plotting ln k_D against the reciprocal absolute temperature, according to the Arrhenius equation, we obtained an activation energy of 72 kJ/mol.

Calculation of Concentration Profiles. In calculation of the complete concentration profiles of mono-, di-, and trimeric MHA free acid the following equations can easily be deduced:

(a) hydrolysis of dimeric MHA free acid (initial conditions: [M] = 0 and [D] = $[D]_0$ at t = 0)

$$[D] = [D]_0 e^{-k_D t} \quad (5)$$

$$[M] = 2[D]_0(1 - e^{-k_D t}) \quad (6)$$

([M] is the concentration of monomeric DL-MHA free acid.)

(b) hydrolysis of trimeric MHA free acid (initial conditions: [M] = [D] = 0 and [T] = $[T]_0$ at t = 0)

$$[T] = [T]_0 e^{-k_T t} \quad (7)$$

$$[D] = \frac{k_T[T]_0}{k_D - k_T}(e^{-k_T t} - e^{-k_D t}) \quad (8)$$

$$[M] = [T]_0\left[3 - 3e^{-k_T t} - \frac{2k_T}{k_D - k_T}(e^{-k_T t} - e^{-k_D t})\right] \quad (9)$$

CONCLUSIONS

The rate constants obtained at 37 °C in 0.1 N aqueous HCl correspond to the following half-lives: $t_{1/2}$ = 1.8 days

for dimeric MHA free acid; $t_{1/2}$ = 1.6 days for trimeric MHA free acid. From eq 5 it can be calculated that it takes 6 days to have 90% of the dimeric MHA free acid split to the monomer (at 37 °C in 0.1 N HCl). These results show that dimeric and trimeric MHA free acids are rather stable toward hydrolysis under physiological conditions of pH and temperature.

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Registry No. (±)-I, 120-91-2; II, 88083-38-9; III, 88083-39-0; HCl, 7647-01-0.

LITERATURE CITED

Boebel, K. P.; Baker, D. H. *Poult. Sci.* **1982**, *61*, 1167–1175.
Cummins, E. W. U.S. Patent 3773927, Nov 20, 1973.
Ingold, C. K. "Structure and Mechanism in Organic Chemistry"; Cornell University Press: Ithaca and London, 1969; pp 1129–1163.
Ivey, F. J., presented at the Delmarva short course in Georgetown, DE, 1981.
Nufer, H. L. U.S. Patent 3272860, Sept 13, 1966.

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Electrophoretic Characterization of Adzuki Bean (*Vigna angularis*) Seed Proteins

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Seed proteins of Minnesota-grown adzuki beans (*Vigna angularis* L. cultivar Takara) were sequentially extracted with deionized distilled H_2O, 0.5 M NaCl, and 0.05 M Tris-HCl buffer (pH 8.6) containing 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol. The protein fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), isoelectric focusing (IEF), and two-dimensional polyacrylamide gel electrophoresis using IEF in the first dimension and SDS–PAGE in the second dimension. The two-dimensional SDS–PAGE electrophoretogram of total seed proteins resolved about 60 polypeptides (spots). The polypeptides of different solubilities were well separated with little or no indication of cross contamination as determined from the electrophoretograms. Periodic acid–Schiff staining revealed two glycoproteins, corresponding to the α and β_1 subunits of the 7S globulin protein. The major seed protein of Takara adzuki bean is a glycoprotein that consists of two carbohydrate-containing subunits with a relative mass (M_r) of 55000 and 35000, respectively.

Adzuki beans have been used in desserts in Oriental countries, especially Japan and China, for over a thousand years. Adzuki beans are used primarily in paste "An" (Japanese for sweetened adzuki bean paste), which is used in desserts, confectionery items, and ice cream and as fillings in baked goods such as Manju and cakes (Hayakawa and Breene, 1982).

In 1977 adzuki beans were evaluated as a potential cash crop in Minnesota. They grow well in Minnesota with a satisfactory yield and excellent An quality. The potential of exporting adzuki beans or their products to Japan or of developing some adzuki bean products that might be acceptable to Western customers has been under serious consideration.

The molecular properties of proteins contribute to the functionality of food ingredients, i.e., specific proteins contribute to the select functional behavior of a food (Cherry et al., 1979). Protein functionality in foods is mainly determined by the molecular composition and structure of the individual proteins (Kinsella and Shetty, 1979). Thus, characterization of the individual seed proteins provides useful information for the isolation and utilization of legume proteins. Although adzuki beans are one of the most important edible legumes in the Oriental countries, little information is available on the properties of their seed proteins. Only the 7S protein of adzuki beans has been well characterized (Sakakibara et al., 1979).

In this study adzuki bean seed proteins were sequentially extracted in three aqueous systems and analyzed by isoelectric focusing and by one-dimensional and two-dimensional polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Dry seeds (11–12% moisture) of adzuki beans (*Vigna angularis* L. cv. Takara) grown in Minnesota were milled so as to pass through a 40-mesh screen.

Protein Extraction. Five grams of ground seed was stirred in 50 mL of doubly distilled water overnight at 4 °C. The slurry was centrifuged at 20000*g* for 30 min at 4 °C and the supernatant carefully removed. The residue was extracted 2 more times for 1 h each at 20 °C. The extracts were pooled and referred to as the water-soluble fraction. Following the third water extraction, the residue was extracted with 50 mL of 0.5 M NaCl (pH adjusted to 7.2 by 0.1 N NaOH) for 1 h with stirring at 4 °C and centrifuged at 20000*g* for 30 min. This extraction was also repeated 3 times. These pooled extracts were referred to as the salt-soluble fraction. The proteins remaining in the residue were extracted by stirring with 30 mL of 0.05 M Tris-HCl buffer (pH 8.6), containing 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol, for 1 h at room temperature (20 °C) and centrifuged at 20000*g* for 30 min to remove the residue. This extraction was repeated 3

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