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A Kinetic Model for Equilibration of Isomeric β-Carotenes†

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The equilibration kinetics and spontaneous isomerization of β-carotenes were studied. Carotene samples were stored at 25 or 45 °C in the dark for varied lengths of time. Isomerization reactions were monitored by HPLC with diode array detection. Two major cis isomers of β-carotene were separated and detected. Results indicated that the extent of isomerization and the proportions of cis isomers formed depended upon the solvent system in which the carotenoid was dissolved. An equilibrium mixture of β-carotenes contained 66.7 ± 3.9% *all-trans*-, 8.2 ± 3.1% 9-*cis*-, and 25.1 ± 2.9% 13-*cis*-β-carotene.

Advances in carotenoid analysis procedures have allowed collection of new information about trans and cis isomers of food carotenes. Determining an exact carotenoid profile, including amounts of various isomers of individual carotenoids in a food, is important for several reasons. The possibility that certain carotenes or their isomers have anticancer potential continues to be investigated (Peto et al., 1981; Watson and Moriguchi, 1985; Watson, 1986; Hennekens, 1986; Beems, 1987). The vitamin A potentials of cis isomers of β-carotene and others are less than those of their all-trans counterparts (Sweeney and Marsh, 1971); therefore, from a bioavailability standpoint it is important to be able to accurately distinguish and quantify the various forms. Also, the colorant properties of carotenoids may be changed significantly with the formation of cis isomers (Zechmeister, 1944). Work by Beadle and Zscheile (1942) demonstrated the conversion of *all-trans*- to *cis*-β-carotene in hexane at 30 °C.

Advances in chromatography have made it possible to separate isomeric forms of carotenoids, and several high-performance liquid chromatography (HPLC) procedures dealing with separation of isomeric β-carotenes have been published (Bushway, 1985, 1986; Khachik et al., 1986; Chandler and Schwartz, 1987; Quackenbush, 1987; Tan, 1988).

Processing and storage conditions can cause isomerization or degradation of carotenoids in foods (Panalaks and Murray, 1970; Sweeney and Marsh, 1971; Ogunlesi and Lee, 1979; Chandler and Schwartz, 1987). However, no information on the exact kinetics of the isomerization reactions in model or food systems has been published.

One objective of this study was to examine the influence of solvent system on spontaneous isomerization of β-carotene with use of common extraction and HPLC mobile-phase solvents. A second objective was to determine the equilibration kinetics of isomerization of *all-trans*-β-carotene.

METHODS

Evaluation of Spontaneous Isomerization. The spontaneous isomerization of β-carotene was evaluated with a working solution of 34 μg/mL β-carotene (type IV) (Sigma Chemical Co., St. Louis, MO) in petroleum ether–acetone (50:50, v/v). Aliquots of 3 mL were evaporated to dryness with nitrogen. Then, the β-carotene was resuspended in 3.0 mL of one of the following solvents: acetonitrile, methanol, tetrahydrofuran (THF, stabilized with 0.025% BHT), chloroform, methylene chloride, or the HPLC mobile phase (acetonitrile–methanol–THF, 42:58:1, v/v/v). The acetonitrile, chloroform, and methanol used were HPLC-grade solvents. Other solvents used in this study were analytical reagent grade.

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Samples were stored in the dark at 25 °C and analyzed for β-carotenes at selected times over a 24-h period. Just prior to analysis, at 1:10 dilution in HPLC mobile phase was made. Samples were analyzed by the HPLC procedure described under chromatographic procedures.

Evaluation of Equilibration Kinetics. The kinetics of β-carotenes equilibration were studied on combinations of single- and multiple-isomer solutions. The source of β-carotenes for this study was a 1% CWS (cold-water-soluble) β-carotene powder (Hoffmann-LaRoche, Inc., Nutley, NJ). This sample was selected because it was determined to contain appreciable amounts of *all-trans*-, 9-*cis*-, and 13-*cis*-β-carotenes. A 4.0-mL aliquot of a working dispersion of 10 μg/mL β-carotenes in distilled water was extracted with use of the basic procedure of Hsieh and Karel (1983). The sample was placed in a 50-mL, screw-capped glass test tube fitted with an aluminum foil sleeve to minimize light exposure. A 10-mL aliquot of petroleum ether–acetone (50:50, v/v) was added to the tube. The tube was mixed, and the liquids were allowed to separate. The ether phase was transferred to another foil-covered test tube. The extraction procedure was repeated until the ether phase was clear. The ether extract then was evaporated to dryness under nitrogen. β-Carotenes were resuspended in 2.0 mL of HPLC mobile phase (acetonitrile–methanol–THF, 42:58:1, v/v/v) and analyzed by the reversed-phase HPLC procedure described under Chromatographic Procedures. Individual β-carotene peaks were collected as they eluted from the exit of the HPLC detector.

Isomeric β-carotene fractions (all-trans, 9-cis, 13-cis) from 12 HPLC runs were pooled, concentrated to a volume of approximately 0.4 mL, and transferred to 1.0-mL reaction vials with Teflon-coated screw caps. The vials were stored in the dark at 45 °C, and the three sets of samples were analyzed for β-carotenes composition at selected times over a 28-day period.

Chromatographic Procedures. HPLC with absorbance detection was used to separate and quantify carotenoid isomers. The HPLC system consisted of a Model 6000A pump (Waters Associates, Inc., Milford, MA), a Model 7125 Rheodyne injector with a 10-μL loop, a Spectroflow 757 variable-wavelength detector (Kratos, Inc., Ramsey, NJ) (used only for fraction collection work), or a Hewlett-Packard 1040 diode array detector equipped with work station computer (Palo Alto, CA). Absorbance at 436 nm was monitored, and scans from 600 to 300 nm were collected throughout the chromatographic runs. Quantitation at 436 nm was used because at this wavelength the three isomeric forms of β-carotene studied all have strong absorbance with similar absorptivities (Stitt et al., 1951). Peak areas of the isomers were compared directly to determine the percentage of each isomer in a mixture. Because absorptivities are not identical, this could result in small inaccuracies for the cis isomers. In the absence of reliable cis standards, an *all-trans*-β-carotene standard was used for quantitation. Carotene separation was accomplished on a Vydac 218TP54 column (Hesperia, CA) with an acetonitrile–methanol–THF (42:58:1, v/v/v) mobile phase at a flow rate of 1.0 mL/min. This mobile phase and column are similar to those reported by Bushway (1986). Peak identification was accomplished against a β-carotene standard and comparison of absorption spectra and retention properties of the isomers with published data.

Data Analysis. Reaction rate constants for the reversible reactions were determined by solving the system of differential equations that represents the kinetics of the isomerization reactions. The parameters of the solutions were determined by nonlinear regression fitting to the experimental data, with use of the Hewlett-Packard 200 series statistical library.

RESULTS AND DISCUSSION

Evaluation of Spontaneous Isomerization. Results of studies on the spontaneous isomerization of *all-trans*-β-carotene showed that minimal quantities of *cis*-β-carotene isomers were present in samples at the beginning of the experiment (Table I). Previous work in our laboratory (Pesek and Warthesen, 1988) showed that extraction and analysis procedures did not induce isomerization; therefore, the small initial quantities of the *cis*-β-carotene isomers in the samples appeared to be present in the β-carotene standard as purchased from Sigma.

Table I. Spontaneous Isomerization of *all-trans*-β-Carotene at 25 °C in HPLC and Extraction Solvents (Values Are Means from Two Experiments)

		% β-carotene isomer		
solvent	time, h	all-trans	9-cis	13-cis
mobile phase	0	94.8	1.7	4.0
	3	97.2		2.8
	6	94.6	2.5	4.4
	24	91.1	2.9	6.0
chloroform	0	97.6		2.5
	3	91.0	4.0	5.1
	6	87.4	5.8	8.0
	24	70.9	15.6	13.6
methylene chloride	0	86.6	2.5	11.0
	3	68.3	8.7	23.1
	6	64.2	11.9	24.0
	24	61.8	15.1	23.2

The THF, methanol, acetonitrile, and HPLC mobile-phase samples contained about 95% *all-trans*-β-carotene and 5% 9-*cis*- and 13-*cis*-β-carotenes at the beginning of the study. After 24 h the β-carotene in the THF, methanol, acetonitrile, and mobile phase samples still contained greater than or equal to 90% *all-trans*-β-carotene. In chloroform, the amount of 13-*cis*-β-carotene increased to 15.6% of the total β-carotenes and the 9-*cis*-β-carotene isomer constituted 13.6% of the total β-carotenes after 24 h. When held in methylene chloride, the amount of 13-*cis*-β-carotene increased to 23.2% of the total β-carotenes and the 9-*cis*-β-carotene isomer constituted 15.1% of the total β-carotenes after 24 h. When the absolute loss of β-carotene was measured, about 20–30% of the initial β-carotene was lost when the mixture was stored in chlorinated solvents for 24 h. These solvent stability data have implications for the selection of solvents for extraction and HPLC mobile phases.

Chromatograms of β-carotene stored in mobile phase or chloroform are presented in Figure 1. The β-carotene stored in the mobile phase showed little change over time, while the *all-trans*-β-carotene sample in chloroform contained approximately 30% cis isomer after 24 h. The rate and/or extent of isomerization of β-carotene was greatest in chlorinated solvents. In general, isomerization was higher in nonpolar solvents than in polar solvents. Zechmeister (1944) reported that the rate of isomerization of β-carotene was higher in toluene (nonpolar) than in nitromethane (a more polar solvent); therefore, this observation holds for additional solvents. Similar results for retinyl palmitate, a compound structurally similar to β-carotene, were presented by Mulry et al. (1983). They found that chlorinated solvents and gold laboratory light promoted isomerization. However, these researchers found that retinyl palmitate was stable in chlorinated solvents when it was stored in the dark. These data indicated that the solvent system in which the carotenoid was dissolved was very important in determining its stability in terms of isomerization.

Evaluation of Equilibration Kinetics. Fractions containing equal to or greater than 83% of 9-*cis*-, 13-*cis*-, or *all-trans*-β-carotene approached a common equilibrium. Starting with 100% *all-trans*-β-carotene, an equilibrium of 66.7 ± 3.9% *all-trans*-, 8.2 ± 3.1% 9-*cis*-, and 25.1 ± 2.9% 13-*cis*-β-carotene was reached after 4–6 days when the mixture was stored in the dark at 45 °C. A three-dimensional chromatogram of a separated equilibrium mixture of β-carotenes is presented in Figure 2. This equilibrium mixture is somewhat similar to that reported

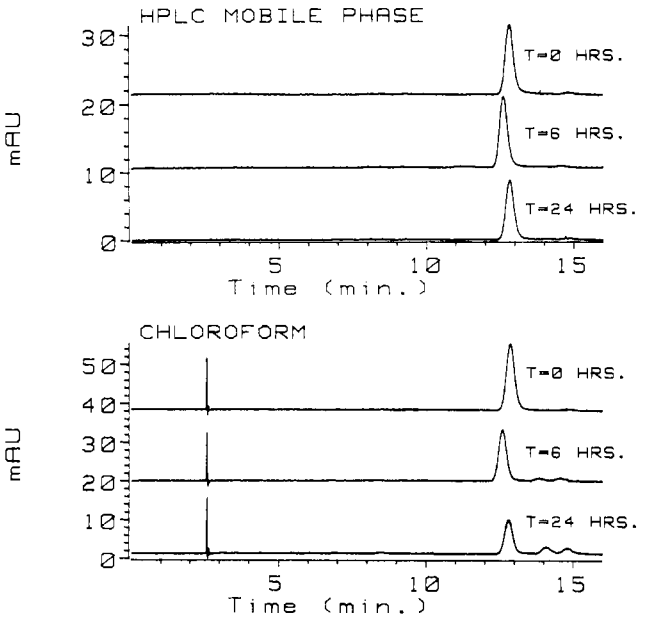

Figure 1. Chromatograms of β-carotene dissolved in HPLC mobile phase and chloroform over time. The large peak at 13 min is *all-trans*-β-carotene. 9-Cis elution is at 14 min and 13-cis at 15 min.

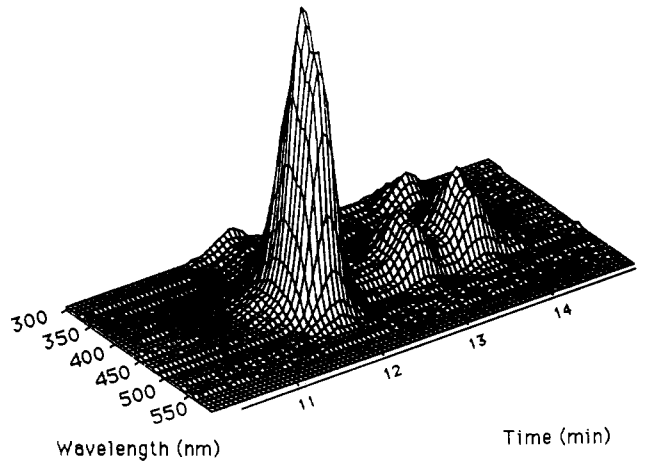

Figure 2. Three-dimensional chromatogram of equilibrium mixture of β-carotenes. *all-trans*-β-Carotene elutes first followed by smaller amounts of 9-*cis*-β-carotene and then 13-*cis*-β-carotene.

by Chandler and Schwartz (1987) for equilibration using I_2 catalysis. These authors reported an equilibrium mixture of 55.9% *all-trans*-, 19.4% 9-*cis*-, and 24.7% 13-*cis*-β-carotene. However, Zechmeister (1944) reported that the composition of an iodine equilibrium mixture usually was different from that of an equilibrium mixture obtained by refluxing of solutions. This difference may include both the ratio of isomers and the presence or absence of detectable amounts of certain isomers.

Although numerous cis isomers theoretically are possible, only two were detected by our HPLC system—9-*cis*- and 13-*cis*-β-carotene. These are the two major cis isomers expected in food systems (Sweeney and Marsh, 1970; Chandler and Schwartz, 1987). Results of studies on the equilibration of isomeric fractions of β-carotenes (collected from the HPLC) indicated that a direct conversion of 9-*cis*- to 13-*cis*-β-carotene or vice versa does not occur. When reaction started with a fraction containing essentially 100% 9-cis, the accumulation of the 13-cis isomer was not observed until after 27% of the 9-cis isomer had converted to the all-trans form. Likewise, when reaction started with a fraction containing 83% 13-*cis*-β-carotene and 17% all-trans, accumulation of the 9-cis isomer was not observed until the all-trans isomer constituted at least 50% of the total β-carotenes concentration.

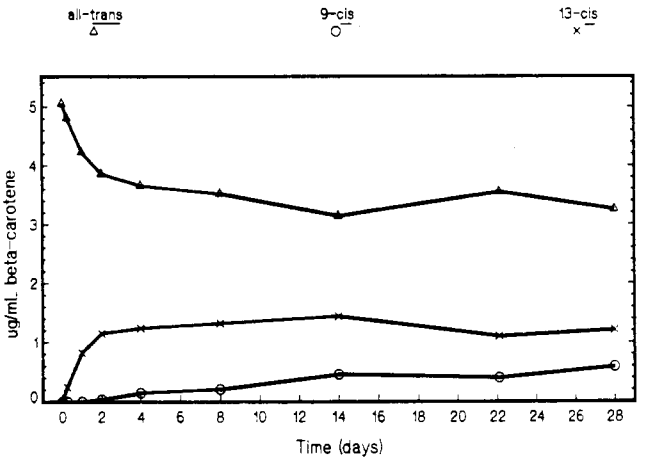

Figure 3. Equilibration of *all-trans*-β-carotene over time in the dark at 45 °C. Each point represents one incubation time.

Table II. Rate Constants for the Equilibration of *all-trans*-β-Carotene in the Dark at 45 °C

	reaction	rate const, day^{-1}
k_1	all-trans to 13-cis	0.23
k_2	13-cis to all-trans	0.61
k_3	all-trans to 9-cis	0.01
k_4	9-cis to all-trans	0.11

Based on the results of studies on the equilibration of isomeric fractions of β-carotenes, a model for the equilibration of β-carotene in solvent mixtures at 45 °C was developed. The model consists of two reversible concurrent reactions

$$\text{9-cis} \underset{k_3}{\overset{k_4}{\rightleftharpoons}} \text{all-trans} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{13-cis} \quad (1)$$

and is represented with the rate equations (2–4), typical of a monomolecular reversible mechanism, where A =

$$\text{all-trans: } dA/dt = -k_1A - k_3A + k_2C + k_4B \quad (2)$$

$$\text{9-cis: } dB/dt = k_3A - k_4B \quad (3)$$

$$\text{13-cis: } dC/dt = k_1A - k_2C \quad (4)$$

concentration *all-trans*-β-carotene, B = concentration 9-*cis*-β-carotene, C = concentration 13-*cis*-β-carotene, t = time, k_1 = rate of conversion of *all-trans*- to 13-*cis*-β-carotene, k_2 = rate of conversion of 13-*cis*- to *all-trans*-β-carotene, k_3 = rate of conversion of *all-trans*- to 9-*cis*-β-carotene, and k_4 = rate of conversion of 9-*cis*- to *all-trans*-β-carotene.

Results of a typical equilibration experiment for β-carotene at 45 °C in the dark are graphically presented in Figure 3. Considering the equilibration model presented and equilibration data, the following trends were noted. With 100% *all-trans*-β-carotene in HPLC mobile phase (acetonitrile–methanol–THF, 42:58:1, v/v/v) under dark conditions, the rate of formation of 13-*cis*-β-carotene was faster than that of 9-*cis*-β-carotene. The back-reaction from the 13-cis isomer to form *all-trans*-β-carotene was slower than the forward reaction, and the formation of *all-trans*- from 9-*cis*-β-carotene was the slowest reaction. These trends do not agree with those reported by Chandler and Schwartz (1987). These authors reported

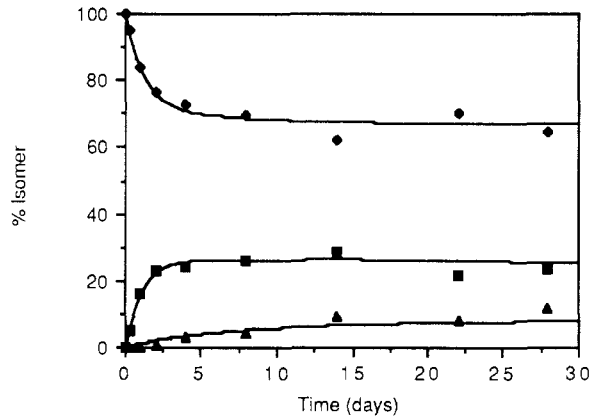

Figure 4. Predicted equations with overlay of experimental data points for equilibration of *all-trans*-β-carotene over time at 45 °C in the dark: (♦) all-trans; (▲) 9-cis; (■) 13-cis.

that the 9-cis isomer was formed as a larger percentage of the total relative to the 13-cis isomer and that it appeared that the 9-cis isomer was kinetically favored. This difference in equilibration could be due to the different solvents or difference in mode of catalysis (spontaneous/solvent in our study vs iodine in the Chandler and Schwartz study).

When the system of differential equations (2–4) is solved, the concentrations of the three isomers can be expressed as a function of reaction time

$$A = G_1 e^{m_1 t} + G_2 e^{m_2 t} + A_{eq} \quad (5)$$

$$B = [k_3 G_1/(m_1 + k_4)]e^{m_1 t} + [k_3 G_2/(m_2 + k_4)]e^{m_2 t} + B_{eq} \quad (6)$$

$$C = [k_1 G_1/(m_1 + k_2)]e^{m_1 t} + [k_1 G_2/(m_2 + k_2)]e^{m_2 t} + C_{eq} \quad (7)$$

where m_1 and m_2 are expressions of the reaction rate constants and G_1 and G_2 are constants that depend on the initial concentration of the mixture. A_{eq}, B_{eq}, and C_{eq} refer to concentrations of the three isomeric forms at equilibrium. When the experimental data were fit to the above equations using nonlinear regression, the reaction rates of the reversible isomerization reactions were determined (Table II).

Equilibration equations for *all-trans*-, 9-*cis*-, and 13-*cis*-β-carotene are presented in eq 8–10, where A = concentration (%) of *all-trans*-β-carotene, B = concentration (%) of 9-*cis*-β-carotene, and C = concentration (%) of 13-*cis*-β-carotene. These equations are dependent upon

$$\text{all-trans: } A = 5.236e^{-0.119t} + 28.064e^{-0.844t} + 66.7 \quad (8)$$

$$\text{9-cis: } B = -7.685e^{-0.119t} - 0.517e^{-0.844t} + 8.2 \quad (9)$$

$$\text{13-cis: } C = 2.449e^{-0.119t} - 27.547e^{-0.844t} + 25.1 \quad (10)$$

the initial concentrations of the isomers in a sample and temperature. In the form presented here they can be used for any combination of initial concentrations. A plot of the predicted equations with an overlay of experimental points is given in Figure 4. This graph illustrates that solutions of the proposed kinetic model fit the experimental data quite well and is evidence that this model provides a good indication of the actual equilibration reactions.

Results of this study indicated that under dark storage conditions at 45 °C β-carotenes reached an equilibrium after 4–6 days. The equilibrium mixture contained approximately 66% *all-trans*-, 8% 9-*cis*-, and 25% 13-*cis*-β-carotene. The rate of formation of the 13-cis isomer was faster than that of the 9-*cis*-β-carotene isomer. The equilibration equations as solved here can be used to predict isomeric composition of a β-carotene sample at any point in time given the specified experimental conditions. With the equations derived here with the proper constants (as determined by experimental parameters), the extent of isomerization in β-carotene systems could be estimated. More complex systems containing β-carotene would have to take into account other rate-influencing factors such as protective effects of the microenvironment, solvents, and other catalysts of isomerization.

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Influence of pH on the Proteinase Complement and Proteolytic Products in Rainbow Trout Viscera Silage

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Nine classes of proteinases were screened for their stability and activity in silages of trout viscera, ensiled at pH 2 and 3. Both endo- and exo-peptidases were active in the pH 3.0 silage (silage A), quickly breaking down the protein nitrogen to amino nitrogen. In the pH 2.0 silage (silage B) only acid endo-peptidase and a weak exo-peptidase activity were present, slowing the formation of amino nitrogen during autolysis. The additional presence of formic acid in pH 2.0 silage (silage C) limited the increase in pH during autolysis and prevented any increase in amino nitrogen, indicating the complete suppression of exo-peptidase activity in such silages. Correspondingly, the amounts of short peptides and amino acids were highest in silage A and smallest in silage C.

The conversion of fish processing wastes and underutilized fish species into fish silage produces a versatile feed ingredient for many farmed animals and fish raised by aquaculture. Although the production of fish silage has become commercialized, the proteolytic enzymes participating in the autolytic process have not been clearly elucidated. Acidic and alkaline proteases have been recovered from silages (Reece, 1988), and the effects of many proteolytic enzymes have been observed in conventional silages (pH 4.0), breaking down proteins to short peptides and free amino acids (Stone and Hardy, 1986). The physical and chemical changes occurring in fish silage during autolysis are well documented (Freeman and Hoogland, 1956; Tatterson and Windsor, 1974; Backhoff, 1976; Raa and Gildberg, 1976; Gildberg and Raa, 1977; Reece, 1980; Stone et al., 1984; Hall et al., 1985; Haard et al., 1985; Stone and Hardy, 1986), but conclusive evidence linking the nature of autolysis to the various proteinases participating in the process has been lacking.

In this study, nine different classes of proteinases were screened for their stability and activity in high- and low-pH silages. Physical and chemical changes occurring in these silages during autolysis were monitored to understand the relationship between the type of proteinases present and the nature of autolysis.

EXPERIMENTAL SECTION

Preparation of Silages. Rainbow trout (*Oncorhymchus mykiss*), 1–2 years of age and weighing between 500 and 800 g (25–40 cm long) were harvested from the aquaculture ponds of the Saskatchewan Research Council, Saskatoon, during Oct 1986. Visceral contents of 100 fish were immediately chilled and later (~5 h) passed through a meat mincer. Minced viscera were thoroughly mixed, frozen in small lots (1 kg) at −40 °C, and stored at −70 °C. Silages were prepared by slowly acidifying the thawed viscera while rapidly mixing with a paddle-type agitator. In silages used for enzyme assays, viscera were initially diluted with water (1.33×) before acidification, to facilitate better extraction and pH adjustment. One group of silages (silage A) were acidified with formic acid (3% w/w) to pH 3.0, while lower pH silages (silage B) were acidified to pH 2.0 with 12 N sulfuric acid. A third type of silage (low-pH buffered silage or silage C) was prepared by further acidifying silage A to pH 2.0 with 12 N sulfuric acid.

Assay of Proteinases. The aqueous phases of silages A and B were used for enzyme assays after 12 h of ensilation at 30 °C. Aqueous phase was obtained by centrifugal separation (2000*g*, 30 min) and filtration of the siphoned aqueous phase through Whatman No. 3 and 42 papers. The pH and protein content (Gornall et al., 1949) of the aqueous phases were measured, and the remaining aqueous phase was stored in aliquots at −70 °C. Aliquots were thawed and then held on ice during the proteinase assays.

General proteolytic activity was assayed at the initial pH of the silages, pH 2 and 3, with azocoll (this and all other proteinase substrates were obtained from Sigma) by a modification of the method of Dean and Domnas (1983). The substrate (5 ± 0.1 mg) was suspended in 2.3 mL of buffer (the compositions of this and other buffers are given in Table I) at 30 °C (all assays were conducted at 30 °C) and the reaction initiated by addition of 0.2 mL of silage. The reaction was terminated after 50 min by adding 1 mL of 35% trichloroacetic acid (TCA) and filtered. Absorbances of filtrates were measured at 520 nm against a water reagent blank. Pepsin-type activity in silage was measured by an identical procedure at pH 1.7, along with a standard pepsin preparation (Sigma; 3× crystallized, 1 mg/mL), and activity expressed as pepsin equivalents. Activities of other pro-

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