APPLICATION OF ACTINIDIN FROM KIWIFRUIT TO MEAT TENDERIZATION AND CHARACTERIZATION OF BEEF MUSCLE PROTEIN HYDROLYSIS

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Received for Publication July 16, 1987
Accepted for Publication November 22, 1987

ABSTRACT

The effect of actinidin on the tenderness of broiled bovine semitendinosus (ST) steaks was studied. An actinidin activity of 400 U/mL resulted in Kramer shear values and sensory tenderness scores equivalent to that produced by Adolph's papain-based meat tenderizer (18 U/mL). Both were significantly ($P<0.05$) more tender than steaks having no tenderizing treatment. Actinidin did not over-tenderize the steak surface as did Adolph's meat tenderizer. Hydrolysis of myofibrillar proteins in enzyme-treated steaks prior to broiling was considerably less for actinidin than for papain when using activities to attain equal Kramer shear values of broiled steaks.

INTRODUCTION

Actinidin is the proteolytic enzyme found in kiwifruit (*Actinidia chinensis*) (Arcus 1959). Even though kiwifruit is a relatively new commodity on the fresh fruit market, the increasing worldwide popularity of kiwifruit has necessitated an expansion of the kiwifruit processing industry to utilize those fruit unacceptable for the fresh market (Dawes 1972). Actinidin for the purpose of meat tenderization may be obtained by direct extraction from the fresh fruit, as a by-product from the preparation of peeled and trimmed frozen pulp and canned fruit or extraction from the juice during ultrafiltration (Wilson and Burns 1983).

It has long been known that raw kiwifruit prevents the solidification of gelatin jellies and this was attributed to a proteolytic enzyme hydrolyzing the gelatin (Arcus 1959). Actinidin is the kiwifruit protease and it contains a free sulfhydryl group, essential for activity (McDowall 1970) and is thus grouped in the class of plant thiol proteases. This group includes papain, ficin and bromelain (Glazer and Smith 1971).

Journal of Food Biochemistry 12 (1988) 147-158. All Rights Reserved.

Crude extracts of actinidin split 20% of the peptide bonds in a 3% gelatin gel in 15 min (1 m*M* cysteine, pH 4.3, 0.25 *M* EDTA) and is more active toward hemoglobin and less active toward peptone and whole pasteurized milk compared with gelatin (Arcus 1959). Bachmann and Farah (1982) demonstrated the occurrence of a bitter taste in mixtures of milk proteins and kiwifruit which was attributed to a very caseinolytic protease in kiwifruit splitting casein into bitter peptides. Yamaguchi *et al.* (1982) showed the caseinolytic protease in kiwifruit had an optimum temperature for activity at 58-62°C, 98% thermal stability at 60°C/10 min and pH stability between 7 and 10.

Actinidin has a broad pH optimum for activity from 5 to 7 (McDowall 1970) using benzoyl-L-arginine ethyl ester (BAEE) as substrate and pH 4.0 to 4.3 for 0.4% gelatin (Arcus 1959). Activity towards BAEE is rapidly lost below pH 3.0 and above pH 10.3. Actinidin has a pI of 3.0 (McDowall 1970) which differs substantially from papain and ficin which have isoelectric points of 8.75 and >9, respectively (Glazer and Smith 1971). Actinidin extracted as a sulfenyl thiosulfate, has a molecular weight of 26,000 and two active proteins are resolved on chromatography on DEAE-cellulose (Boland and Hardman 1972). Carne and Moore (1978) determined the molecular weight to be 23,500 by amino acid sequence of the enzyme. McDowall (1973) fractionated actinidin, extracted in its active form, on DEAE-Sephadex A25 (pH 5.0) and found two active components, each homogeneous on PAGE and designated them as A_1 and A_2 in order of increasing electrophoretic mobility towards the anode.

The objective of this study was to assess the meat tenderizing ability of actinidin and characterize the molecular changes to beef muscle proteins due to its proteolytic activity.

MATERIALS AND METHODS

Isolation of Actinidin from Kiwifruit

Kiwifruit, grown in Winters, California, was obtained 2 days postharvest and ripened for three weeks at 5°C (actinidin activity and content increases with fruit ripeness; Lewis and Luh 1988). Actinidin was extracted as the fully active enzyme by the method of Brocklehurst *et al.* (1981) but without covalent chromatography. The extract was precipitated (60% saturated $(NH_4)_2SO_4$) and resuspended in 0.1 *M* KH_2PO_4/NaOH buffer, pH 6.0 containing 1 m*M* EDTA and 50% sat. $(NH_4)_2SO_4$. This resulted in an enzyme suspension which retained its activity when stored at 4°C for at least 2 months. This stock solution contained 4.15 mg protein/mL and 540 U/mL (specific activity 130 U/mg) at pH 6.0.

Actinidin and Papain Activity Assays

Actinidin and papain activities were monitored throughout by spectrophotometric assay (Boland and Hardman 1972). This involved using the substrate N-α-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester (CBZ-lys-ONp) and measurement of increased absorbance at 348nm due to *p*-nitrophenol production at pH 6.0 using a Varian DMS 100 UV/Visible spectrophotometer with DS-15 kinetics storage program. Due to some ionization of the *p*-nitrophenol at acid pH, it was necessary to incorporate a hydrolysis correction factor for the pH used in the assay (eg; factor=15.36 at pH 6.0, based on pK_a=7.16 for *p*-nitrophenyl). Protein content was monitored throughout by either absorbance at 280 nm or the method of Lowry *et al.* (1951).

The substrate CBZ-lys-ONp (12 m*M*) was prepared in 19:1 acetonitrile to water (v/v). The 0.07 *M* phosphate buffer contained 0.2 m*M* EDTA and 1.6% acetonitrile at pH 6.0. Papain stock solution (Type III, Sigma Chemical Co.) was diluted to 12 u*M* in 0.05 *M* acetate buffer, pH 5.2, to make a working solution. Substrate solution (50 μl) was added to 2.98 mL of 0.07 *M* phosphate buffer (pH 6.0) and spontaneous hydrolysis of the ester substrate recorded for 40 s (A_{348}). Enzyme solution (50 uL) was then added and the change in A_{348} (dA/dt) recorded for a further 260 s. The rate of nonenzymatic substrate hydrolysis was then subtracted from the enzymatic dA/dt and activity expressed in units of μmoles product formed per min using $\Delta\epsilon$=5400 $M^{-1}cm^{-1}$ (Boland and Hardman 1972).

Preparation of Enzyme Solutions

All enzymes were prepared in 0.1 *M* phosphate reaction buffer, pH 6.0, containing 1 m*M* EDTA and 1 m*M* cysteine and the control was reaction buffer without enzyme.

Adolph's Meat Tenderizer

The recommended concentration of the commercial meat tenderizer, 2.5 g for 200 g steak, was prepared in 30 mL reaction buffer, centrifuged (18,000 x *g*, 5°C, 20 min) and the supernatant protease activity determined.

Papain

Papain (type III, Sigma Chemical Co.), used in the study as a standard, was adjusted to an enzyme activity equivalent to Adolph's Meat Tenderizer in the reaction buffer.

Actinidin

The actinidin suspension was first adjusted to an enzyme activity equivalent to that found in Adolph's tenderizer to determine if this would result in a similar degree of tenderization. The actinidin activity was then adjusted to a series of activities

(18, 100, 140, 170 and 430 U/mL) by dilution in reaction buffer to determine the optimum level of actinidin activity for effective tenderization. This level was then compared with Adolph's Meat Tenderizer using papain as a standard.

Meat Tenderization

Eight ST steaks of 2.5 cm thickness were removed from left and right sides of the twelfth rib area of two fully mature cows (>3 years, USDA Good grade carcass) at 48 h postmortem. Meat samples were kindly supplied by Y.B. Lee of the Department of Animal Science, Univ. of California, Davis, CA 95616. All visible fat was removed from the steaks and they were stored at −20°C in sealed polyethylene bags until needed for tenderization. Each set of four weighed steaks was equilibrated to room temperature (ca.20°C) and the internal temperature measured at the geometric center with copper/constantine thermocouples and a Molytek 32 channel recorder/data logger (Molytec, Inc., Pittsburgh, PA.). Samples of beef steak (approx. 200g which had been forked 10 times each side to a depth of about 1.5 cm) were incubated with 30 mL of either actinidin, papain, Adolph's tenderizer or control solutions (reaction buffer) for 30 min, turning the steaks four times to ensure equal time exposure to the enzyme solutions. Steaks were then broiled to an internal temperature of 70°C (ca.10-15 min). Cooked steaks were weighed and samples from each steak were removed for Kramer shear testing and sensory analysis.

Measurement of Shear

The modified Kramer shear test (Lee 1983) was used as an objective assessment of tenderness of the broiled steaks. The correlation with sensory panels has been reported to be of the same magnitude for both methods (Pearson 1963). Samples for sensory analysis were removed from the broiled steaks using a cutting template made from plexiglass to form uniform samples of dimensions 2.8 x 2.8 x 2.5 cm. The remaining meat from each steak sample was pooled and ground through a Hobart (N50) electric food grinder (with an extrusion head with openings of 6 mm diameter). The ground meat from each steak was mixed and triplicate 20 g samples were shear-tested (20°C) using the Kramer shear device (6.7 mm x 6.7 mm test cell) which was mounted on an Instron Universal Testing Machine, with a 200 kg head at a crosshead speed of 20 cm/min. Shear values were expressed as the average kg force per 20 g sample.

Sensory Analysis

The sensory evaluation of tenderness was performed by five panel members on 8-point intensity scale; 1=extremely tender, 2=very tender, 3=moderately tender, 4=slightly tender, 5=slightly tough, 6=moderately tough, 7=very tough, 8=extremely tough. Five panel members (20-30 years, 2 male and 3 female) were selected by screening individuals to assess whether they could reproducibly

distinguish between very tough (intensity 7; Kramer shear 130 kg/20g) and very tender (intensity 2; Kramer shear 40 kg/20g) meat samples as estimated by Kramer shear values. The panel was asked to compare and rate each of the four treated broiled steaks (actinidin, Adolph's, papain and control) which were heated in a microwave oven and served at 70°C internal temperature in covered insulated boxes. Sensory testing was repeated on two alternate days in the late morning in booths under red lights. The sensory results were analyzed by a three-way analysis of variance using the Statistical Analysis System (SAS Institute Inc. 1984) taking into account treatment, judge and day effects and the t-Test between means calculated to generate least significant differences ($P<0.05$).

Actinidin and Papain Digestion of Beef Myofibrillar Protein and Myosin Extract

Bovine ST muscle tissue (1.0 g) from the same carcasses used in the meat tenderization analyses was stripped of all visible fat and homogenized with 0.05 *M* Tris buffer (9 mL), pH 7.0, at 4°C for 1 min in a Waring Blendor and centrifuged for 10 min at 2,000 x *g*. The myofibrils were washed twice in 0.05 *M* Tris buffer, pH 7.0, and finally suspended in 0.1 *M* phosphate buffer, pH 6.0, containing 1 m*M* dithiothreitol and 1 m*M* EDTA. This extract was digested with actinidin at 300 U/mL and with papain at 18 U/mL for 15 min at 22°C. The digested extracts were adjusted to 1.0 mg protein/mL with sample buffer (0.01 *M* Tris.HCl, 0.001 *M* EDTA, 1% SDS, 5% β-mercaptoethanol, 0.02% bromphenol blue, pH 8.0); heat denatured (10 min at 100°C) and cooled before analysis on SDS polyacrylamide gel electrophoresis (PAGE). Samples (20 μL) were loaded into SDS PAGE slab gels of linear concentration gradient (5-25% acrylamide) and run at 25 mA for 5 h (modified from Laemmli 1970).

Native myosin heavy chain (HC) was a gift from Dr. E. Bandman and was prepared by the method used by Shelton and Bandman (1985). The myosin HC preparation contained 2.3 mg protein/mL in 0.1 *M* phosphate, pH 6.0, 1 m*M* EDTA and 1 m*M* dithiothreitol enzyme reaction buffer. Myosin HC was digested by actinidin (400 U/mL) in a time course reaction at 22°C. Samples were removed at 0, 5, 10, 15 and 20 min and immediately mixed with 1/2 volume sample buffer and heat denatured as for the muscle extracts. The myosin digests were analyzed by SDS PAGE (Laemmli 1970) using 10% gels.

RESULTS AND DISCUSSION

Meat Tenderization

The mean shear values for meat tenderization by actinidin at the suggested Adolph's papain-based meat tenderizer concentration are summarized in Table 1. Actinidin had no effect upon tenderization when used at the same activity as Adolph's meat tenderizer (18 U/mL). This was unexpected since Boland and Hardman (1972)

TABLE 1.
MEAN SHEAR VALUES FOR MEAT TENDERIZED AT SUGGESTED ADOLPH'S MEAT TENDERIZER CONCENTRATION

SAMPLE	ACTIVITY[a] (U/ml)	SHEAR[b] (Kg/20g)
CONTROL	0	115
ADOLPH'S	18	91
ACTINIDIN	18	114
PAPAIN	18	87

[a]Esterase activity towards CBZ-lys-ONp.
[b]Average of three 20 g samples of ground steak.

determined that the activity of actinidin towards CBZ-lys-ONp was similar to papain. We compared the enzyme specificity for CBZ-lys-ONp of our actinidin preparation with papain and found it to be only 25% as specific as papain (Lewis and Luh 1988). This difference could be attributed to our use of an enzyme extraction method which was different from Boland and Hardman and the kiwifruit strain used in our study together with environmental affects could have resulted in a different specificity.

From Fig. 1 , an actinidin activity greater than 175 U/mL resulted in shear levels

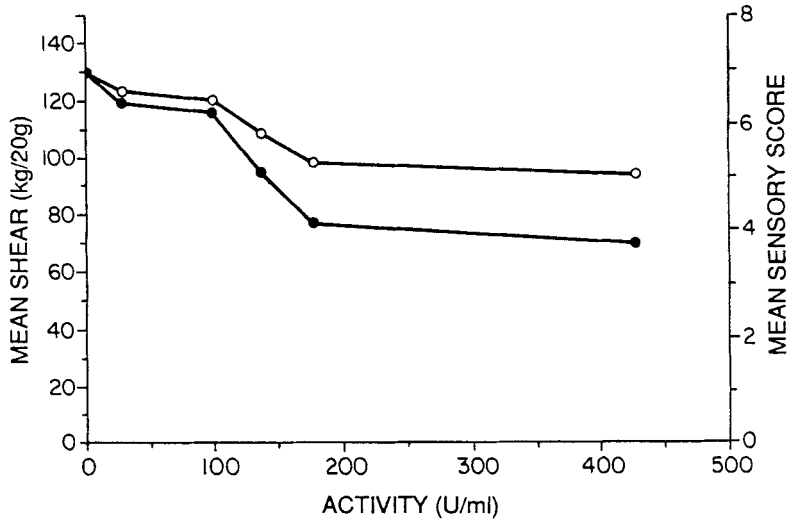

FIG. 1. EFFECT OF ACINIDIN ACTIVITY ON KRAMER SHEAR (n=3) AND SENSORY TENDERNESS INTENSITY (5 judges x 2 reps.) OF BROILED BEEF STEAK

comparable to those obtained using Adolph's meat tenderizer where a Kramer shear value of 91 kg/20 g for Adolph's meat tenderizer and 87 kg/20 g for papain, both at 18 U/mL. Actinidin activity levels greater than 300 U/mL produced shear and sensory scores which were significantly more tender than the control steaks (95% confidence level). Tenderization by actinidin did not produce off flavors or odors in the meat or excessive surface tenderization.

The comparison of actinidin tenderized steaks with papain tenderized steaks is shown in Table 2. There was no difference between the mean sensory scores for tenderness for the enzyme-treated samples but there was a difference between the control sensory scores and the enzyme-treated samples (at the 95% confidence level). The sensory panel noted that some of the steaks treated with Adolph's meat tenderizer and papain tended to have a "mushy" texture at the surface but were still firm internally. Actinidin did not produce surface "mushiness". These results suggested that actinidin hydrolyzed fewer peptide bonds in the muscle proteins.

SDS PAGE of Muscle Hydrolysis Products

The effects of actinidin and papain on the myofibrillar protein extract from beef muscle are shown in Fig. 2. The control (lane A) shows the extracted native muscle proteins and corresponding protein identity. Lane B shows the action of actinidin (300 U/mL) on the control muscle extract. The myosin band in the control muscle

TABLE 2.
COMPARISON OF ACTINIDIN MEAT TENDERIZATION WITH PAPAIN TENDERIZED BEEF STEAKS

SAMPLE	ACTIVITY (U/ml)	MEAN SHEAR (Kg/20g)	MEAN SENSORY TENDERNESS INTENSITY (5 judges X 2 reps.)
CONTROL	0	120[a]	6.7[a]
ADOLPH'S	18	85	5.0
ACTINIDIN	400	84	5.5
PAPAIN	18	81	5.4

[a]significantly different from means of Adolph's, actinidin and papain at the 95% confidence level.

FIGURE 2.

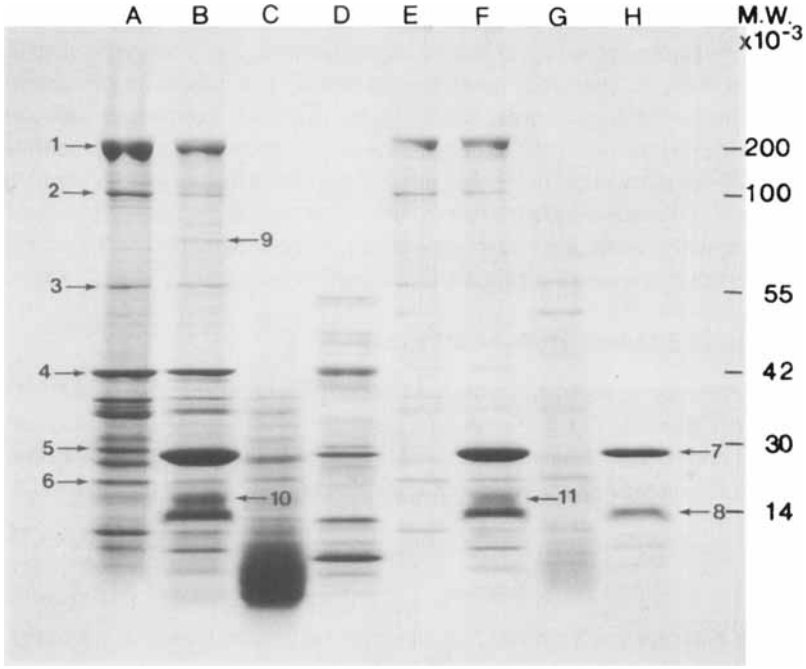

FIG. 2. ACTION OF ACTINIDIN AND PAPAIN ON MYOFIBRILLAR PROTEIN EXTRACT FROM BEEF MUSCLE
(SDS PAGE, linear gradient 5-25% polyacrylamide).
LANE: A:insoluble muscle protein extract; B:(A) +actinidin (15 min, 20°C, pH 6.0, 300 U/mL, 5 mg/mL); C:(A)+papain (15 min, 20°C, pH 6.0, 18 U/mL); D:molecular weight markers; E:myosin (2.5 mg/mL); F:myosin+actinidin (300 U/mL); G:myosin+papain (18 U/mL); H:actinidin (2.5 mg/mL).
BAND: 1. myosin HC; 2. α-actinin; 3. desmin; 4. actin; 5. myosin LC1; 6. troponin C; 7. actinidin A_1; 8. actinidin A_2; 9. 69,000D; 10. and 11. 21,000D.

extract was reduced by 43% in this reaction (based on relative peak areas from densitometry). Most of the control protein bands were reduced by actinidin. Two new bands appeared in the actinidin/muscle protein reaction at points 9 and 10, corresponding to molecular weights of about 69,000 D and 21,000 D, respectively. The 21,000 D band also appeared in the native myosin HC/actinidin reaction (point 11, lane F).

Lane C shows the action of papain at 18 U/mL on the muscle extract. All of the myosin disappeared as did most of the other muscle protein bands. One band appeared near the myosin LC1 with a molecular weight about 22,000 D. A similar band did not appear in the myosin/papain reaction (lane G).

Lane D contains the molecular weight markers: lysozyme, carbonic anhydrase, soybean trypsin inhibitor, phosphorylase B, bovine serum albumin, and myosin HC. Lane E contains the myosin HC extract, showing some hydrolysis products. Lane F shows the effect of actinidin at 300 U/mL on the myosin HC extract in Lane E. As mentioned above, a band corresponding to 21,000 D appeared which was also found in the actinidin/muscle protein reaction. Another strong band appeared at 15,500 D in the actinidin/myosin HC reaction which was present in the muscle extract control and the actinidin/muscle reaction.

Lane G shows the action of papain at 18 U/mL on the myosin HC extract of Lane E. All the myosin was degraded with the production of low molecular weight products from 17,000 D to 14,000 D. It is interesting to note that the two bands in the myosin extract (lane E) with apparent molecular weights of 20,700 D and 16,600 D were not hydrolyzed by papain. Lane H shows two bands of actinidin (bands 7 and 8) A_1 and A_2, respectively, as also found by McDowall (1973).

At these activities, actinidin did not hydrolyze as many myofibrillar proteins as papain but resulted in the same degree of meat tenderization as measured by Kramer shear values and sensory analysis (Table 2). This may explain why actinidin-tenderized steaks did not develop surface over-tenderization. Actinidin may be specific in hydrolyzing muscle proteins particularly important in meat toughness rather than hydrolyzing all proteins which may alter textural properties such as mouthfeel, as does papain.

SDS PAGE of Myosin Hydrolyzed by Actinidin

Figure 3 shows an SDS polyacrylamide electrophoretogram (10% gel) and Fig. 4 shows the densitometry scans for the time course hydrolysis of native myosin HC by actinidin (450 U/mL) for 25 min at 20°C. The two forms of actinidin are seen in the gel with molecular weights of A_1 23,000 D, and A_2 14,000 D.

At least four additional bands with molecular weights 138,000 D, 120,000 D, 101,000 D and 84,000 D appeared after 5 min reaction. This pattern resembles the observations by King *et al.* (1981) that papain decreased the myosin heavy chain with the concurrent increase in material in the C-protein and α-actinin regions on SDS PAGE of various combinations of different protein fragments. C-protein is a component of the thick filaments of the myofibrills and has been reported as acting to help hold the thick filament during tension development, influence cross-bridges in muscle contraction and affect actin/myosin interaction (Robson *et al.* 1982). The solubilization of C-protein prior to cooking may affect meat tenderness.

FIGURE 3.

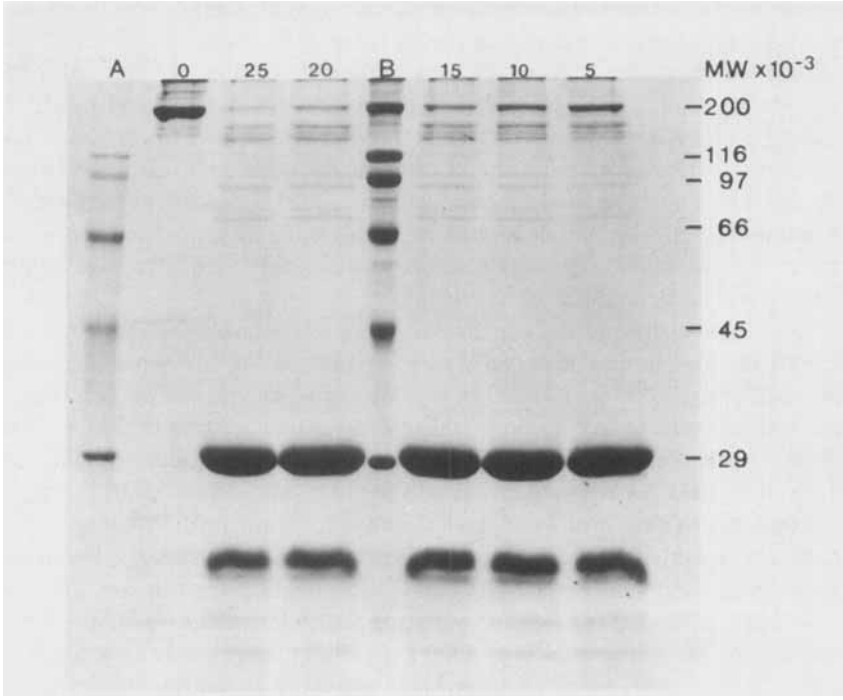

FIG. 3. SDS PAGE OF ACTION OF ACTINIDIN ON NATIVE MYOSIN
(pH 6.0, 0.1 *M* phosphate buffer, 1.0 m*M* cysteine, at 450 U/mL, for 0, 5, 10, 15, 20 and 25 min, 25°C). Lanes A and B (double conc.) are the standard molecular weight markers (myosin, β-galactosidase, phosphorylase b, bovine albumin, egg albumin, carbonic anhydrase; Sigma Chemical Co.).

The "21,000 D" band which appeared in Fig. 2 was not visible in Fig. 3, probably because it ran with the A_1 band of actinidin on the 10% gel in which the actinidin concentration was higher (450 U/mL compared with 300 U/mL in the muscle hydrolysis reaction in Fig. 2) and the single concentration gel does not give the same degree of resolution possible with the linear gradient gel.

From the densitometry scan of the 25 min reaction time (Fig. 4), low molecular weight proteins were produced ranging from 10,000 D to 7,000 D. These probably resulted from myosin breakdown rather than from actinidin autoproteolysis because the two actinidin peak areas remained constant throughout the 25 min reaction. Actinidin was most efficient in the tenderization of beef steaks at activities ranging from 300 to 400 U/mL at pH 6.0 (using CBZ-lys-ONp as substrate). Actinidin

hydrolyzed myofibrillar proteins less than papain and does not produce over-tenderization at the meat surface for the same degree of overall tenderness. Thus the extent of actinidin hydrolysis can be controlled by the time of application prior to cooking the meat.

It has been shown that partially purified actinidin will tenderize beef steaks; however the purified enzyme is an expensive means of utilizing the fruit for its meat tenderizing capabilities. Alternatively, the partially purified enzyme could be included in a papain- or bromelain-based powder or liquid product so that less of the more active enzymes papain or bromelain would be needed. This may reduce undesirable surface over-tenderization and would represent a novel product. A fresh-fruit puree, bottled and stored refrigerated may also be a feasible commercial product requiring minimal processing. Further research in this direction would benefit the consumers and kiwifruit processing industry.

FIGURE 4.

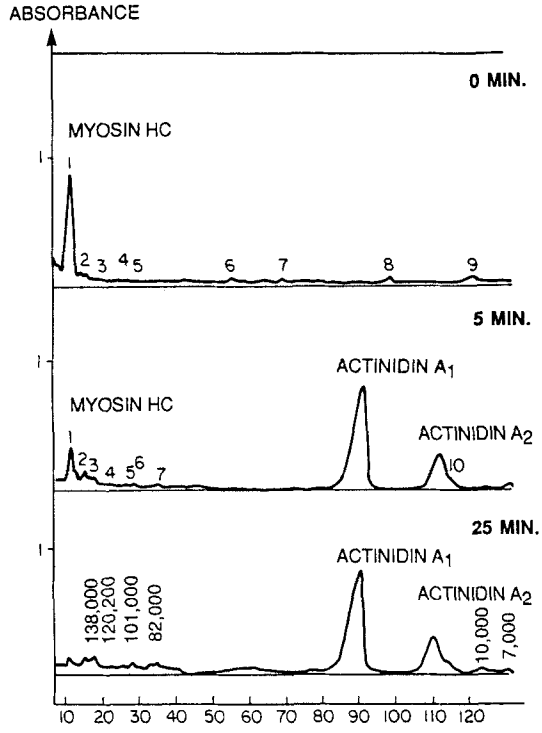

FIG. 4. DENSITOMETRY SCANS FOR THE SDS PAGE OF ACTINIDIN HYDROLYSIS OF NATIVE MYOSIN

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