BACTERIOCINS PRODUCED BY STRAINS OF

LACTOBACILLUS ACIDOPHILUS ISOLATED

FROM DIFFERENT ANIMAL SPECIES

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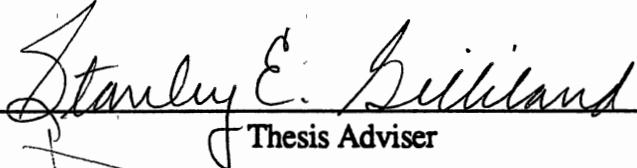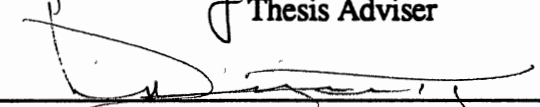
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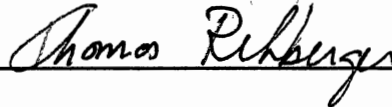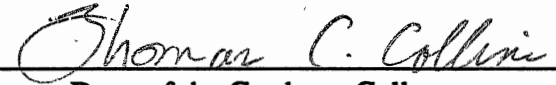Thesis
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CHAPTER I

INTRODUCTION

Antagonistic effects produced by lactobacilli toward other organisms have been recognized since the report of Metchenikoff (1908). These inhibitory effects produced by lactobacilli may play an important role in maintaining a proper microbial balance in intestinal tract (Sandine, 1979) and preserving certain foods (Daeschel, 1989).

Major inhibitory factors produced by lactic acid bacteria include lactic acid and/or other organic acids produced by lactobacilli as a result of fermentation (Gilliland, 1985a). Formation and accumulation of hydrogen peroxide during growth also can cause inhibition of other microorganisms including intestinal and foodborne pathogens (Gilliland and Speck, 1977). Other toxic metabolites including D-leucine (Gilliland and Speck, 1968) and diacetyl (Jay, 1982) can be inhibitory factors.

Bacteriocins are protein or protein related complexes with bactericidal mode of action directed against species that are usually closely related to the producer bacterium (Tagg et. al., 1976). Since Rogers (1928) first indicated the presence of antibiotics among lactic acid bacteria, a number of lactobacilli including strains of *Lactobacillus acidophilus* have been shown to produce bacteriocins or bacteriocin-like inhibitory substances (Klaenhammer, 1988). Most bacteriocins are heat stable and sensitive to certain proteolytic enzymes. The inhibitory spectra of bacteriocins generally is restricted to closely related species of bacteria. However, some bacteriocins have been found active against foodborne pathogens including *Listeria monocytogenes*(Schillinger and Luke, 1989; Ahn and Stiles, 1990a; Hoover et. al., 1988 Pucci et. al., 1988; Nielson et. al., 1990), and

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Clostridia species (McCormick and Savage, 1983; Daeschel and Klaenhammer, 1985; Muriana and Klaenhammer, 1991a).

Many attempts have been made to purify bacteriocins produced by lactobacilli using various methods including ammonium sulfate precipitation, gel chromatography, ion exchange chromatography, and high pressure liquid chromatography. Bacteriocins can occur as complexes with lipid and carbohydrate (Barefoot and Klaenhammer, 1984, Upreti and Hinsdill, 1973; Muriana and Klaenhammer, 1991a). Molecular weights of bacteriocins have been estimated as small as 1.7 KDa (Piard et. al., 1992) to 42 KDa (Rammelsberg et. al., 1990).

The genetic determinants of bacteriocin production and host immunity can be either chromosomally controlled (Barefoot and klaenhammer, 1983; Muriana Klaenhammer, 1987 and 1991b; Joerger and Klaenhammer, 1986 and 1990) or plasmid borne (Schillinger and Luke, 1989; Graham and McKay, 1985; Daeschel and Klaenhammer, 1985; Gonzalez and Kunka, 1987).

The objectives of this study were: 1) to assay the bacteriocin activity produced by various strains of *Lactobacillus acidophilus* isolated from intestinal source of different origins including humans, pigs, calves, chickens, and rodents; 2) to isolate and purify the bacteriocin(s) and investigate their properties to determine whether there were differences in bacteriocins produced by various strains of *L. acidophilus*.

CHAPTER II

REVIEW OF LITERATURE

Antagonistic Activity of Lactic Acid Bacteria

Metchnikoff (1908) speculated that the acid producing lactobacilli could suppress the harmful bacteria normally occurring in the intestinal tract. A number of studies and reviews since then have been reported the factors related to the inhibitory effect of lactic acid bacteria (Hurst, 1973; Babel, 1977; Gilliland, 1985a; Ferreira and Gilliland, 1988; Gilliland, 1989; Daeschel, 1989).

A major inhibitory factor produced by lactic acid bacteria is acid, which lowers the pH of environment to the point where other bacterial growth may be inhibited (Gilliland, 1985a; Ferreira and Gilliland, 1988). In fermented food, reduction of pH resulting from acid production by fermentation is the primary preserving actions of these lactic acid bacteria (Daeschel, 1989). The type of acid produced by lactic acid bacteria is very important in regard to the intensity of the antagonistic action they produce (Gilliland, 1985b). For example, acetic acid was more inhibitory toward the undesirable microorganisms than an equal concentration of lactic acid (Sorrells and Speck, 1970). Acid producing lactobacilli also appeared to be important in helping maintain a proper balance among microorganism in the intestinal tracts (Sandine et. al., 1972; Speck, 1976; Sandine, 1979). However, the intensity of the antagonistic action may not be directly related to the amount of acid produced (Gilliland and Speck, 1972; Gilliland and Speck, 1977; Mitchell and Gilliland, 1983). It also has been recognized that lactic acid bacteria

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are capable of producing inhibitory substances other than organic acids (Gilliland, 1985a and 1985b; Daeschel, 1989).

Many lactic acid bacteria have the ability to produce hydrogen peroxide under aerobic conditions (Dahiya and Speck, 1968; Gilliland and Speck, 1969; Price and Lee, 1970; Collins and Aramaki, 1980). Accumulation of hydrogen peroxide in growth media can occur because lactic acid bacteria do not possess the enzyme catalase (Kandler and Weiss, 1986). The formation and accumulation of hydrogen peroxide has resulted in autoinhibition of some lactic acid bacteria (Gilliland and Speck, 1969; Keen, 1972; Stanley, 1977) and inhibition of other microorganisms such as *Staphylococcus aureus* (Wheater et. al., 1952; Dahiya and Speck, 1968), *Pseudomonas* spp.(Price and Lee, 1970), and intestinal and foodborne pathogens (Gilliland and Speck, 1977).

The accumulation of toxic metabolites other than hydrogen peroxide also can be important in controlling growth of bacteria (Gilliland, 1985b). Gilliland and Speck (1968) reported that D-leucine produced by lactic streptococci was inhibitory to the culture. A metabolic end product diacetyl (2,3-butanediol) has been shown to be inhibitory to gram-negative and non-lactic acid Gram-positive bacteria (Jay, 1982).

Rogers (1928) first indicated the presence of antibiosis among lactic acid bacteria. Considerable efforts thereafter have been made to characterize the antibiotic substances (Babel, 1977). In 1944, Oxford extracted an inhibitory agent from cultures of *Streptococcus cremoris* and called the substance diplococcin. Mattick and Hirsh (1944) concentrated the inhibitory substance produced by *S. lactis* which completely inhibited several organisms even in considerably diluted solution. In their later report (Mattick and Hirsh, 1947), the substance was designated as nisin, derived from Group N streptococci. Tagg et. al. (1976) defined these inhibitory substances as bacteriocins of Gram-positive bacteria. Bacteriocins are protein or protein related complexes with bactericidal mode of action directed against species that are usually closely related to the producer bacterium (Tagg et. al., 1976; Klaenhammer, 1988).

Properties of Bacteriocins

Since Vincent et. al.(1959) characterized lactocidin, an antimicrobial agent produced by *Lactobacillus acidophilus* from various sources, bacteriocins have been characterized as proteinaceous compounds having bactericidal mode of action. The inhibitory protein produced by *L. acidophilus* AC_1 was a single peptide which was active over a wide range of pH and heat sensitive (Metha et. al., 1983). Barefoot and Klaenhammer (1983 and 1984) defined lactacin B produced by *L. acidophilus* N2 as a bacteriocin which was sensitive to proteolytic enzymes, and retained full activity after 60 min at $100^{o}C$ at pH 5. Lactacin F produced by *L. acidophilus* 88 was heat stable and sensitive to proteases (Muriana and Klaenhammer, 1987). The production of lactacin F was pH dependent in that it was produced at highest level in MRS broth cultures maintained at pH 7.0, whereas negligible bacteriocin activity was detected at pH 5.0 or 7.5.

Other species of lactobacilli also have been reported to produce bacteriocins. Wheater et. al. (1951) reported that lactobacillin was produced by a strain of homofermentative *Lactobacillus helveticus*. They found, however, that hydrogen peroxide was involved in the inhibitory mechanisms, and withdrew the name 'lactobacillin' (Wheater et. al. 1952). Upreti and Hinsdill (1973) revealed that lactocin 27 was produced by *L. helveticus* LP27 among the strains of lactobacilli obtained from human and animal sources. They showed that lactocin 27 inhibited primary protein synthesis without affecting DNA, RNA, or ATP synthesis in a later study (Upreti and Hinsdill, 1975). Lactocin 27 appeared to act on the cell membrane and was adsorbed in a nonspecific way by the cells. Helveticin J, a bacteriocin produced by *L. helveticus* 481, was active at neutral pH under aerobic and anaerobic conditions, and was sensitive to proteolytic enzymes and heat (30 min at $100^{o}C$). It also demonstrated a bactericidal mode of action against sensitive indicator

strains. Production of helveticin J was maximized in an anaerobic fermentor held at a constant pH of 5.5 (Joerger and Klaenhammer, 1986)

De Klerk and Coetzee (1961) described antagonistic activities among the strains of lactobacilli isolated from many different sources including human saliva. The properties of inhibitory substance were different from those of phage suspensions. The activity was diffusible in agar and was not affected either by boiling for 15 min or by adjusting to pH 7. Activity was not diminished by the presence of catalase ruling out the possible direct involvement of hydrogen peroxide. In a later study, De Klerk (1967) concentrated the inhibitory activity by ammonium sulfate precipitation and confirmed the inhibitory activity as bacteriocins. De Klerk and Smit (1967) further characterized the bacteriocin produced by *Lactobacillus fermenti* 466 and demonstrated that the inhibitor was sensitive to trypsin and pepsin, but resistant to treatment with either heat (96° C for 30 min), urea or lysozyme. The bacteriocin was a macromolecular lipocarbohydrate protein and the biological activity of the complex was dependent on its structural integrity.

Bacteriocins produced by *Lactobacillus plantarum* have been reported and characterized. Daeschel et. al. (1986) reported production of platacin A by a strain of *L. plantarum* isolated from cucumber fermentation. Platacin A was bactericidal, proteinaceous, heat stable (30 min at 100° C), active over pH range from 4.0 to 6.5 and retained in dialysis membranes permeable below 8,000 Da. West and Warner (1988) suggested that platacin B produced by *L. plantarum* NCDO 1193 was a protein complexed with carbohydrate and/or lipid. Andersson et. al. (1988) reported the binding of plantaracin SIK-83, a bacteriocin produced by *L. plantarum* SIK-83 to the cell was specific for sensitive cells.

Sakacin A (Schillinger and Luke, 1989) and lactocin S (Mortvedt and Nes, 1990), bacteriocins produced by strains of *Lactobacillus sake*, were proteinous in nature and demonstrated bactericidal mode of action. Lactocin S was moderately heat-stable and its activity was found in the growth medium during the late exponential phase of growth.

Lactobacillus sp. strain 100-37 isolated from murine gastrointestinal tract produced a bacteriocin (McCormick and Savage, 1983). The activity was not inducible with mitomycin C or UV light, but was stable in flowing steam (100°C) for up to 50 min, in buffers over a range of pH 1.6 to pH 6.8. It was nondialyzable and inactivated by trypsin and papain.

The bactericidal activity of the inhibitor produced by *Lactobacillus casei* ssp. *rhamnosus* GR-1 was retained when the cell free spent broth was adjusted to pH 7.0. The inhibitory substance was heat labile, not precipitated by up to 80% ammonium sulphate, and extractable in chloroform. The inhibitor was not lactic acid or hydrogen peroxide (McGroarty and Reid, 1988). Rammelsberg et. al. (1990) reported that *L. casei* B 80 synthesizes a mitomycin C inducible polypeptide (caseicin 80) with very specific bactericidal activity against the sensitive strain. The amount of secreted bacteriocin in the culture solution was low, about 111 mg/l. The bacteriocin also was detectable in cell extracts, although only 2% of total activity was retained intracellularly. More recently, Toba et. al. (1991a and 1991c) reported production of bacteriocins from *Lactobacillus delbrueckii* subsp. *lactis* JCM 1106 and 1107, and strains of *Lactobacillus gasserii* isolated from infant feces.

Several other researchers have reported production of heat-stable proteinaceous bacteriocins by species of lactobacilli and other lactic acid bacteria. These includes bacteriocins from *Lactobacillus bulgaricus* RS 902 (Sinha, 1991), *Lactobacillus brevis* B37 (brevicin: Rammelsberg and Radler, 1990), *Bifidobacterium* (Meghrous et. al., 1990), *Carnobacterium piscicola* LV17 isolated from vacuum-packed meat (Ahn and Stiles, 1990), *Leuconostoc gelidium* (Harding and Shaw, 1990; Harding and Stiles, 1991), *Leuconostoc mesentroides* UL5 (Daba et.al., 1991), and *Propionibacterium thoenii* (Lyon and Glatz, 1991).

Since Etchells et. al. (1966) reported that growth of *L. plantarum* was severely retarded in brined cucumber fermentations when the inoculum included *Pediococcus*

cerevisiae FBB-61, bacteriocins from the strains of pedicocci have been studied extensively. Fleming et. al. (1975) found that inhibitory agent other than acid or hydrogen peroxide was produced by *P. cerevisiae* FBB-61, and suggested that the production of this inhibitor might explain the appearance of pediococci during the first stage of natural fermentations of brined cucumbers and Spanish-type green olives. The antagonistic activity of this inhibitory agent was inactivated by pronase, but was resistant to heat treatment at 100° C for 60 min (Rueckert, 1979).

Gonzalez and Kunka (1987) reported pediocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0 was bactericidal in its mode of action and sensitive to various proteolytic enzymes. Pucci et. al. (1988) examined the action of pediocin PA-1 against *Listeria monocytogenes* using a dried powder prepared from *P. acidilactici* PAC 1.0 culture supernatant fortified with 10% nonfat milk powder. Inhibition occurred over the pH range 5.5 to 7.0 and at both 4 and $32^OC.$ Later, Gonzalez (1989) obtained a patent for an invention relating to the use of bacteriocin produced by *P. acidilactici* PAC 1.0 to inhibit bacterial spoilage by Gram positive bacteria, particularly lactobacilli in a food system (refrigerated salad dressing).

Pediocin AcH, an antimicrobial peptide produced by *P. acidilactici* H was characterized first by Bhunia et. al. (1988). The bacteriocin was sensitive to proteolytic enzymes, resistant to heat and organic solvents, and active over wide range of pH. Bhunia et. al. (1990) examined the toxicity of pediocin AcH and reported that the bacteriocin was nonimmunogenic in immunizing mice and a rabbit. It was also non-toxic to laboratory animals and was hydrolyzed by gastric proteolytic enzymes. They suggested that the nature of pediocin AcH may be considered favorable in its possible use as a food preservative. Biswas et. al. (1991) reported the maximum production of pediocin AcH by *P. acidilactici* H in a broth medium (pH 6.5) within 16 to 18 h at 30 to 37^OC (final pH, 3.6 to 3.7). Pediocin AcH production was negligible when the pH of the medium was maintained at 5.0 or above, even in the presence of high cell mass. Yousef et. al. (1991)

reported that in the presence of pediocin AcH or a derivative of *P. acidilactici* H, JBL 1095, there was appreciable decrease in numbers of *Listeria monocytogenes* in exudative fluids from beef wieners throughout the storage period at 4°C and 25°C. This suggests the possible use of bacteriocins to control this pathogen in foods.

<div align="center">Inhibitory Spectrum of Bacteriocin Activity</div>

The spectra of activity of bacteriocins produced by most lactic acid bacteria are limited to closely related species of bacteria (Klaenhammer, 1988; Tagg et. al., 1976). Most of bacteriocins produced by *L. acidophilus* displayed narrow inhibitory spectra (Vincent et. al., 1959; Barefoot and Klaenhammer, 1983; Muriana and Klaenhammer, 1987; Muriana and Klaenhammer, 1991a). Metha et. al.(1983), however, reported that the protein produced by *L. acidophilus* AC$_1$ was active against both Gram-positive and Gram-negative bacteria. Helveticin J (Joerger and Klaenhammer, 1986) and lactocin 27 (Upreti and Hinsdill, 1973), bacteriocins produced by *L. helveticus,* were active against closely related species.

A number of studies have demonstrated the antagonistic activity of bacteriocins of lactic acid bacteria against *L. monocytogenes* in addition to other lactic acid bacteria. Schillinger and Luke (1989) reported bactericidal activity of sakacin A, a bacteriocin produced by *L. sake* Lb706, against *L. monocytogenes. Carnobacterium piscicola* LV17 (Ahn and Stiles, 1990a), *Leuconostoc gelidium* (Harding and Shaw, 1990), and *Leuconostoc mesentroides* UL5 (Daba et. al., 1991) also produced bacteriocins active against *L. monocytogenes.* Lactic acid bacteria isolated from various sources exhibited bacteriocin activity against *L. monocytogenes.* These sources include goats' milk (Hechard et. al., 1990), vacuum-packaged fresh meat (Ahn and Stiles, 1990b), and retail cuts of meat (Lewus et. al. 1991). Strains of *Pediococcus* species produced bacteriocins active against *L. monocytogenes*(Hoover et. al., 1988; Bhunia et. al., 1988; Pucci et. al., 1988; Spelhaug and Harlander, 1989; Nielsen et. al., 1990; Yousef et. al., 1991). Recently,

Foegeding et. al. (1992) indicated that pediocin produced by *P. acidilactici* PAC 1.0 was responsible for part of inactivation of *L. monocytogenes* during fermentation and drying process of sausage production.

Inhibitory activity of bacteriocins against other pathogenic and/or spoilage bacteria related to food also have been reported. McCormick and Savage (1983) detected the antagonistic effect by *Lactobacillus* sp. strain 100-37 isolated from murine gastrointestinal toward an obligate anaerobic *Clostridium ramnosum* H1 isolated from mouse feces. Pediocin A produced by *P. pentosaceus* FBB61 also was shown to be effective against *Clostridium botulinum, C. perfringens,* and *C. sporogenes* in addition to strains of *Staphylococcus aureus* and *Streptococcus lactis* (Daeschel and Klaenhammer, 1985: Spelhaug and Harlander, 1989). Bhunia et. al. (1988) reported that pediocin AcH produced by *P. acidilactici* H was active against *C. perfringens* and *S. aureus.* *Enterococcus faecalis* was reported to be sensitive to a bacteriocin produced by *Leuconostoc gelidium* (Hastings and Stiles,1991), and to lactacin F produced by *L. acidophilus* 11088 (Muriana and Klaenhammer, 1991a).

Nisin exhibited a relatively wide range of inhibition of Gram positive bacteria including strains of streptococci, staphylococci, micrococci, and lactobacilli (Broughton, 1990). It has been effective particularly against spore formers such as *C. botulinum* (Taylor et. al., 1990; Scott and Taylor, 1981), and *C. sporogenes* (Rayman et. al., 1981). *L. monocytogenes* also was sensitive to inhibitory action of nisin (Daeschel et. al., 1990; Williams and Tatini, 1990; Benkerroum and Sandine, 1988). Recently, Stevens et. al. (1991) reported the inactivation of *Salmonella* by nisin when used in combination with chelating agent EDTA.

Purification and Estimated Molecular Weight of Bacteriocins

Vincent et. al. (1959) purified lactocidin produced by *L. acidophilus.*

A 2,500 fold purification was achieved by the chromatography with silicic acid. Purified lactocin was unstable and was inactive in serum. Metha et. al. (1983) indicated that the inhibitory protein produced by *L. acidophilus* AC$_1$ was a single polypeptide with a molecular weight of 5.4 KDa. Barefoot and Klaenhammer (1983) reported that molecular weight of lactacin B produced by *L. acidophilus* N2 was approximately 100 KDa for the crude inhibitor. They later purified lactacin B by ion-exchange chromatography, ultrafiltration, and successive gel filtration on Sephadex G-75 in the presence of 8 M urea and then 0.1% sodium dodecyl sulfate (Barefoot and Klaenhammer, 1984). The molecular weight of lactacin B was ca. 6.0 to 6.5 KDa and the purified compound showed maximum absorbance at 211 nm. Muriana and Klaenhammer (1991a) isolated lactacin F produced by *L. acidophilus* 11088 as a floating pellet from a broth culture supernatant brought to 35 to 40% saturation with ammonium sulfate. The size of crude lactacin F was identified as 180 KDa by gel chromatography. Electron microscopic examination of the active fraction showed micelle-like globular particles. Further purification by ammonium sulfate precipitation, gel filtration, and high performance liquid chromatography resulted in a 474 fold increase in specific activity of bacteriocin. The purified bacteriocin was identified as a 2.5 KDa peptide by SDS polyacrylamide gel electrophoresis (SDS-PAGE). The lactacin F activity was retained after extraction from SDS-PAGE gel slices. Composition analysis indicated that lactacin F may contain as many as 56 amino acid residues.

In 1973, Upreti and Hinsdill reported that lactocin 27 produced by *L. helveticus* LP27 was isolated and purified from the culture supernatant fluid as a protein-lipopolysaccharide complex by the series of steps including chloroform precipitation and Sephdex G-200 column chromatography with and without SDS. In the presence of SDS the complex was dissociated, and the activity was found to reside in a small glycoprotein with molecular weight of 12.4 KDa. Joerger and Klaenhammer (1986) reported that helveticin J, an antimicrobial agent produced by *L. helveticus* 481, was present as an aggregate with a molecular weight in excess of 300 KDa in its crude form. Helveticin J

was purified by ammonium sulfate precipitation followed by gel filtration chromatography in the presence of SDS. SDS-PAGE of purified helveticin J resolved a 37,000 Da protein band with bacteriocin activity.

De Klerk and Coetzee (1961) first indicated the inhibitory activity produced by *L. fermenti* was not extractable with ether but was precipitated by saturated ammonium sulfate and also by ethanol. Later, De Klerk (1967) purified the bacteriocin by dialysis, chromatography on Sephadex G-100 and calcium phosphate gel column. The bacteriocin was a macromolecular lipocarbohydrate protein which consists of 16 amino acids, 4 sugars, hexosamine and phosphorus (De Klerk and Smit, 1967).

Lactocin S, a bacteriocin produced by *L. sake* L45, was purified to homogeneity by ion exchange, hydrophobic interaction and reverse phase chromatography, and gel filtration (Mortvedt et. al., 1991). The purification resulted in approximately a 40,000-fold increase in the specific activity of lactocin S and enabled the determination of a major part of its amino acid sequence. Exclusion of the nonionic detergent, Tween 80, from the MRS broth resulted in a high, reproducible recovery of lactocin S in the ammonium sulfate precipitation rather than foam or floating fraction as reported by Muriana and Klaenhammer (1991a). The amino acid composition indicated that lactocin S consisted of 33 amino acids, of which about 50% were the nonpolar amino acids alanine, valine, and leucine. The 25 residue C-terminal part of lactocin S was sequenced and demonstrated highly non-polar characteristic. Three unidentified residues of 25 C-terminal sequence were possibly modified forms of cystein and/or amino acids associated with cystein in a manner similar to that seen in lanthionine residues present in nisin. The hydrophobic nature of lactocin S and its homology with signal sequences suggested that the cell membrane as a possible target for lactocin S.

Rammelsberg et. al. (1990) concentrated caseicin 80 produced by *L. casei* B80 by ultrafiltration and purified it by cation exchange chromatography. The molecular weight was in the range of 40 to 42 KDa and the isoelectric point was pH 4.5. Daba et. al. (1991)

examined mesenterocin 5 produced by *Leuconostoc mesentroices* UL5 and found that the bacteriocin activity corresponded to an apparent molecular weight of about 4.5 KDa by SDS-PAGE.

Lyon and Glatz (1991) partially purified propionicin PLG-1 produced by *Propionibacterium thoenii* from solid medium by ammonium sulfate precipitation (60% saturation). It was purified further by gel filtration on Sephadex G-200. The results of gel filtration showed that bacteriocin was present as two protein aggregates with one having an apparent molecular weight of more than 150 KDa and the other approximately 10 KDa. Resolution of these protein aggregates by SDS-PAGE revealed the presence of a protein common to both with an apparent molecular weight of 10 KDa. The molecular weight of pediocin PA-1 produced by *P. acidilactici* PAC1.0 (Gonzalez and Kunka, 1987) and pediocin AcH produced by *P. acidilactici* H (Bhunia et. al. 1988) have been identified as 16.5 KDa and 2.7 KDa , respectively.

Piard et. al. (1992) purified lactacin 481, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CNRZ 481, by ammonium sulfate precipitation, gel filtration, and HPLC. The entire purification resulted in a 107,506 fold increases in the specific activity. The molecular weight was estimated as 1.7 KDa. Dimers of 3.4 KDa also exhibited bacteriocin activity.

Mode of Action of Bacteriocins

In 1975, Upreti and Hinsdill examined the effect of lactocin 27 produced by *L. helveticus* LP27 on the sensitive cells. It inhibited primarily protein synthesis without affecting DNA and RNA synthesis or ATP synthesis level. However, it caused a leakage of potassium ions and an influx of sodium ions, which suggested that it acted on the cell membrane. The adsorption of lactocin 27 was non-specific. Andersson et. al. (1988) reported more detailed study of inhibitory mechanism of plantaracin SIK-83 produced by *L. plantarum* SIK-83. The binding of bacteriocin to the cell was specific to sensitive cells.

Sensitive cells, after exposure to the bacteriocin, could be rescued by treatment with proteolytic enzymes. In buffer, plantaricin SIK-83 was adsorbed to the cell surface almost immediately, and morphological lesions were observed within 2 h after the cells were exposed to the bacteriocin. They suggested that the lethal mode of action took place in 2 steps: a comparatively rapid attachment of the bacteriocin to the cell surface, followed by killing of the cells and cell lysis. The bacteriocin acts, directly or indirectly, by damaging the cell membrane.

Law and Dajani (1978) studied the inhibitory mechanism of viridin B, a bacteriocin produced by *Streptococcus mitis*. Oxygen consumption by actively growing cultures of *Niseria sicca* ceased immediately upon exposure to viridin B. ATP production was slightly enhanced within 1 h of exposure to the bacteriocin but was subsequently repressed. The uptake and incorporation of glucose was prevented in the presence of viridin B. The bacteriocin also blocked uptake of an amino acid mixture in cells pretreated with chloramphenicol. Although viridin B blocked protein and nucleic acid synthesis, no degradation of such macromolecules was observed. The inhibitory effect on macromolecular synthesis and on viability required the presence of sufficient nutrients to allow active metabolism. The bacteriocin did not inhibit viability or macromolecular synthesis in aerobically incubated cultures. On the basis of these findings, they proposed that the bacteriocin inhibits actively growing *N. sicca* by disruption of membrane-bound components responsible for oxygen-dependent electron transport, which in turn uncouples energy transduction necessary for accumulation of precursors. Cells which are not actively metabolizing or are metabolizing anaerobically utilize other methods of energy transduction which are unaffected by viridin B. Tagg and Wannamaker (1978) reported that the streptococcin A-FF22 produced by group A *Streptococcus* strain FF22 was associated with the cell walls of producer strain, and the nature of binding was nonspecific and was attributed to electrostatic interaction. More recently, Zajdel et. al. (1985) investigated the mechanism of bactericidal activity of lactostrepcin 5, a bacteriocin

produced by *Streptococcus cremoris* 202. The bacteriocin did not kill protoplasts of sensitive cells, and its activity was decreased about 10-fold after pretreatment of the cells with trypsin, suggesting the involvement of the cell wall in the activity of the bacteriocin. In susceptible cells, the bacteriocin slowed down and then stopped synthesis of DNA, RNA, and protein. Lactostrepcin also inhibited uridine transport in susceptible cells and induced leakage of K^+ and ATP. Survival of cells treated with the bacteriocin in phosphate buffer was higher in the presence of K^+, Ca^{2+}, or Mg^{2+}. They suggested that the primary target for the bacteriocin was a modification of permeability of the cell envelope. The bacteriocin effect on ATP, K^+, and possibly also Ca^{2+} and Mg^{2+} render the cells unable to preserve their integrity and maintain the intracellular pH; therefore, deterioration of intracellular energy metabolism occurs, leading to cell death.

The mode of action of nisin is one of most extensively studied among bacteriocins from Gram-positive bacteria (Broughton, 1990). The point of action of nisin on the vegetative cells is the cytoplasmic membrane. Its action causes disruption, either resulting in leakage of essential cellular material or in more severe cases lysis. The disruption is caused by nisin inactivating sulphydryl groups in the cytoplasmic membrane(Morris et. al., 1984). Nisin action against spores is sporadic rather than sporostatic. Nisin inhibits the germination process at the stage of pre-emergent swelling (Hitchins et. al., 1963). Nisin had been shown to inhibit murein (peptidoglycan) synthesis (Reisinger et. al., 1980). This inhibition was caused by the formation of a complex between the antibiotic and lipid intermediate. Ruhr and Sahl (1985) indicated that nisin affected cytoplasmic membrane permeability properties. Nisin was also shown to cause a rapid efflux of amino acids and radioactively labeled Rb^+ (K^+ analog) from the cytoplasm of Gram-positive bacteria. They concluded that the cytoplasmic membrane was the primary target and that membrane disruption accounts for the bactericidal action of nisin. Kordel et. al. (1989) reported that the cationic peptide nisin along with antibiotics Pep 5 and subtilin depolarized bacterial and artificial membranes by formation of voltage-dependent multi-state pores. The peptide

antibiotics did not span bilayer membranes in the absence of a membrane potential and therefore that the potential should be necessary to force the peptides into a transmembrane orientation during pore formation. Liu and Hansen (1990) demonstrated that the dehydroalanine and dehydrobutyrine residues in active nisin play an important role by reacting with nucleophiles in the cytoplasmic membrane of a sensitive cell. Recently, Gao et. al. (1991) studied the interaction of the peptide antibiotic nisin with liposomes. Nisin dissipated the membrane potential and the pH gradients. It also inhibited oxygen consumption by cytrochrome c oxidase. The dissipation of proton motive force was only to a minor extent due to a decrease of the oxidase activity. The membrane potential and/or pH gradient across the membrane enhances the activity of nisin. Nisin incorporates into the membrane and makes the membrane more permeable for ions. As a result, both the membrane potential and pH gradient are dissipated. The activity of nisin was found to be influenced by the phospholipid composition of the liposomal membrane.

Although Gram-negative bacteria are not generally sensitive to nisin (Hurst, 1981), some researchers have studied methods to interrupt outer cell membrane of Gram-negative bacteria, so that the bacterium can be more susceptible to antibiotic agents. Kordel and Sahl (1986) showed that *Esherichia coli* exhibited nisin sensitivity when the outer membrane was altered by treatments such as osmotic shock. Blakburn et. al. (1990) proposed that nisin can be used in combination with a chelating agent, surfactant, and/or other bacteriocins to enhance a bactericidal effect towards both Gram-positive and Gram-negative bacteria. Recently, Stevens et. al. (1991) reported that nisin in combination with chelating agent, disodium EDTA, was bactericidal to *Salmonella* species and other Gram-negative bacteria.

Genetic Determinants of Bacteriocins

Chromosomal

Barefoot and Klaenhammer (1983) attempted first to determine genetic determinants of bacteriocin production and host immunity in strains of *L. acidophilus*. Plasmid DNA was not detected in *L. acidophilus* N2, suggesting that production of lactacin B may be chromosomally controlled. Muriana and Klaenhammer (1987) reported that the lactacin F production and immunity was not related to the 4 and 27 MDa plasmids detected in the *L. acidophilus* 88. The conjugal transfer of determinants of the bacteriocin production and immunity implicated that the determinants of both genes were chromosomal, and transferred by the form of plasmids that reintegrated into the chromosome of the recipient following transfer. In a recent study, Muriana and Klaenhammer (1991b) showed the cloning, expression, and nucleotide sequence of a gene encoding a lactacin F production and immunity. A oligonucleotide probe specific for the lactacin F structural gene (*laf*) was synthesized based on the findings from the former study (Muriana and Klaenhammer, 1991a). Cloning experiment revealed that a 2.2 Kb *Eco* R1 fragment of a plasmid DNA (pTRK 162) of a lactacin F-producing transconjugant related to the *laf* gene, and this was confirmed by transformation via electroporation of pTRK 162 into lactacin F-negative strains. An 873-bp region of the 2.2 kb fragment was sequenced and the analysis of the resulting sequence identified an open reading frame (ORF) which could encode a protein of 75 amino acids. The 25 N-terminal amino acids for lactacin F were identified within the ORF along with an N-terminal extension of 18 amino acid residues, possibly a signal sequences. They suggested that the remainder of 57 amino acid residue (6.3 KDa) portion of *laf* gene corresponds well to composition analysis of purified lactacin F thus indicating that the bacteriocin may contain as many as 56 amino acid residues (Muriana and Klaenhammer, 1991a).

L. *helveticus* 481 was subjected to plasmid curing experiments and the results provided no evidence for a plasmid harboring determinants for helveticin J production nor for host immunity (Joerger and Klaenhammer, 1986). Later, Joerger and Klaenhammer (1990) reported the DNA sequence of a contiguous 3,364-bp region on the chromosomal DNA from *L. helveticus* 481 for helveticin J production. Two complete open reading frames (ORF), designated ORF2 and ORF3, were identified within the sequence. They suggested that ORF 3 could encode a 37,511-Da protein, whose molecular weight would be close to that of helveticin J (37,000 Da) as estimated from a previous study (Joerger and Klaenhammer, 1986). ORF 2, which potentially encodes a 11,808-Da protein, might be responsible for the immunity protein that binds to helveticin J and facilitates its export from the cell. The recombinant plasmid pTRK135 containing ORF2 and ORF3 was transformed into *L. acidophilus* via electroporation. Transformants produced a bacteriocin with same characteristic as that of helveticin J.

Plasmid Borne

Schillinger and Luke (1989) indicated that a plasmid of about 18 MDa may be involved in the formation of sakacin A, a bacteriocin produced by *L. sake* Lb706 as well as in host immunity. Lactocin S production and immunity (*L. sake* L45) also were reported to be related with a 50 Kb plasmid (Mortvedt and Nes, 1990). Ahn and Stiles (1990a) indicated the production of and resistance to the bacteriocin produced by *Carnobacterium piscicola* LV17 were associated with two plasmids of 40 and 49 MDa. Hastings and Stiles (1991) examined a bacteriocin produced by *Leuconostoc gelidium* and determined that the loss of 7.6 MDa plasmid resulted in loss of production and resistance to the bacteriocin.

Graham and McKay (1985) demonstrated that the production of the bacteriocin correlated with the presence of a 10.5 MDa plasmid in *P. cerevisiae* FBB-63. It was not clear, however, if immunity to the bacteriocin was associated with this or other plasmids.

Daeschel and Klaenhammer (1985) examined the bacteriocin-producing strains of *P. pentosaceus* for plasmid content and genetic stability of bacteriocin production and host cell immunity. They suggested that the loss of bacteriocin production and host cell immunity was closely linked on an unstable genetic determinants and the loss of these phenotypes was irreversible. Plasmid analysis identified 13.6 MDa plasmid (pMD136) which encoded both bacteriocin immunity and production. Gonzalez and Kunka (1987) provided evidence that the production of pediocin A (*P. acidilactici* PAC1.0) was associated with the presence of a 6.2 MDa plasmid. Hoover et. al. (1988) reported that the bacteriocin activity was harbored on a plasmid approximately 5.5 megadalton in three strains of *P. faecalis* and one strain of *P. pentosaceus* isolated from fermented sausage. Ray et. al. (1988) reported that pediocin AcH (*P. acidilactici* H) activity and immunity phenotypes were correlated with a 7.4-MDa plasmid (pSMB74). In their later study, Ray et. al. (1989) suggested that the occurrence of pSMB74 in Bac^+Bac^r transconjugants, obtained from conjugal mating between the donor *P. acidilactici* H and the recipient *P. acidilactici* LB42-315, established that this plasmid encoded both Bac^+ and Bac^r determinants.

CHAPTER III

MATERIALS AND METHODS

Sources and Maintenance of Cultures

The cultures used in this study were obtained from the stock culture collection from the Dairy Food Microbiology Lab, Department of Animal Science at the Oklahoma State University, Stillwater. All cultures of lactobacilli were maintained by subculturing in lactobacilli MRS broth (DIFCO laboratories, Detroit, MI) using 1% inocula and 18 to 20 h incubation at 37°C. The cultures were stored at 1-2°C between transfers. Prior to use in experiments, each culture was subcultured at least three times. For long term storage, the cells were harvested by centrifugation from MRS broth culture and resuspended in MRS broth containing 20% glycerol (Fisher, Pittsburgh, PA). The suspension was stored at - 70°C.

Cultures of *Listeria monocytogenes* Scott A and V_7, and *Esherchia coli* 0157:H7 (ATCC 43895) were maintained in Brain Heart Infusion (BHI; Difco) broth and in Trypticase Soy Broth (TSB; Difco), respectively. Other procedures for propagating and maintaining these cultures were the same as stated as for *L. acidophilus*.

Preparation of Cell Free Spent Broth

MRS broth cultures incubated at 37°C for 18 h were harvested by centrifugation (8,000 x g, 10 min). The supernatant fluids were collected, and adjusted to pH 6.5 with 10 N NaOH (Sigma, St. Louis, MO). The spent broths were filtered through sterile 0.45 μm

acrodisc filters (Gelman, Ann Arbor, MI) into sterile screw cap test tubes. The cell free spent broth was stored at refrigeration temperature (1^oC).

Screening for Bacteriocin Production

Agar Plate Assay

A five ml portion of MRS agar inoculated (1%) with the desired indicator culture was poured into a 110 x 10 mm petri dish. After solidification, 10 µl portions of spent broths from producer cultures were aseptically placed on the surface of the seeded agar. The petri dishes were incubated upright for 24 h at 37^oC. The presence of inhibitory material in the spent broth samples was indicated by clear inhibitory zones on the agar.

Inhibitory activity of the samples obtained from purification steps were assayed against *Lactobacillus delbrueckii* subsp. *lactis* 4797 as a indicator strain throughout the study.

Serial Dilution Assay

A serial dilution assay was utilized to determined the relative amounts of inhibitory activity. The samples were diluted in a series of twofold serial dilutions using sterile distilled water. A 10 µl portion of each dilution was placed on the surface of seeded agar and incubated as stated in the agar plate assay procedure. The highest dilution which prevented growth was recorded and the reciprocal of the dilution was defined as the arbitrary inhibitory activity unit (AU).

Effect of Heat on Inhibitory Action of the Spent Broth

The inhibitory samples of spent broth were heated at 121^oC for 15 min, cooled to room temperature and assayed for inhibitory activity by the agar plate assay.

Effect of Catalase and Proteases on Inhibitory Action

To ascertain whether or not the inhibitory activity was due to hydrogen peroxide or protein, the following enzymes and pH values were used : A) Catalase (pH 6.0; E.C. NO. I. II. I. 6) from bovine liver, B) Trypsin (pH 8.0 ; Type II, crude), and C) Pepsin (pH 3.0; E.C. NO. 3.4.23.I). All enzymes were obtained from Sigma Chemical Company (St. Louis, MO)

Twenty five milligrams of each enzymes was added to 5 ml of cold distilled water, vortexed to dissolve, filtered through a sterile acrodisc filter (0.45μm, Gelman) into a sterile tube and kept in ice water for immediate use. Spent broth from the producing strains was adjusted to each pH value and filtered through sterile 0.45 μm filters. Ten ml aliquots of spent broth at each pH level were placed into sterile tubes and 0.5 ml of proper enzyme or distilled water as control was added. All tubes were incubated in a waterbath at 37°C for 30 min. Following incubation, each sample was adjusted to pH 6.0 with 1N of HCl or NaOH as required, filtered through a sterile acrodisc filter (0.45μm) again and assayed for bacteriocin activity by agar plate assay. The amounts of alkali and/or acid added were measured and compensated with distilled water so that the final volumes of all tubes were equal. If catalase had no effect and proteolytic enzymes eliminated the inhibitory activity, it was assumed to be due to bacteriocin(s).

Ammonium Sulfate Precipitation

The proper amount of ammonium sulfate (Sigma) was added slowly to spent broth from cultures that produced bacteriocin to make the solution 50% saturated with ammonium sulfate. The precipitated fraction was harvested by centrifugation at 10,000 x g for 20 min at 1°C and resuspended in 2 ml of 0.05 M Tris-HCl buffer (pH 8.0), with or without 0.1% sodium dodecyl sulfate (SDS; Sigma) as desired. The fractions were vortexed until completely dissolved and collected for further purification. The collected

samples were dialyzed overnight at 4°C with stirring using No. 2 dialyzing tubing (M.W. cut off, 12 to 14 KDa; Spectrum, Los Angeles, CA) against two liters of the same buffer.

<center>Sephadex G-200 Gel Column Chromatography</center>

Three grams of Sephadex G-200 (Pharmacia, Piscataway, NJ) powder per 100 ml of column volume was added slowly to warm distilled water (70°C) with continuous stirring. The solution was heated for 5 h at 70 - 80°C and then cooled at room temperature. After washing the gel with five times the column volume of distilled water two to three times, the gel was equilibrated with 0.05 M Tris-HCl buffer (pH 8.0), with or without 0.1% SDS as appropriate, overnight at 4°C. The slurry was poured into a 2.5 x 50 cm glass column (Bio-Rad, Richmond, CA) and enough volume of the buffer was eluted until the gel was settled completely. The final gel volume in the column was 200 ml. The column was equilibrated with two to three times its volumes of buffer. A 1% Blu Dextran 2000 (Sigma) was used to ensure the homogeneity of column packing and to determine the void volume. General conditions of gel chromatography was practiced as recommended by Stellwagen (1990).

The column outlet was attached to a pump (Cole-Parmer, Chicago, IL) which was set to a flow rate of 0.23 ml/min. The sample of approximately 4% of total column volume was loaded onto column and eluted with the buffer. The eluent was collected in 5 ml of fractions and was monitored for absorbance at 280 nm. Each fraction was assayed for bacteriocin activity by the serial dilution method as described previously. Inhibitory activity and $A_{280\ nm}$ were plotted against elution volumes to locate protein fractions that were inhibitory.

Sodium azide (0.02%, Fisher) was added to the buffer to prevent microbial growth in the column for long term storage. Before a sample was loaded, more than two times the column volume of buffer was passed through the column to remove the sodium azide and

the eluted buffer was examined for inhibition by the agar plate assay to ensure the complete removal of the sodium azide.

Microconcentration

Contents of tubes containing each inhibitory fraction eluted from gel chromatography were pooled and concentrated with Centricell 60 concentration unit (Polysciences, Warrington, PA) with molecular retention limit of 30,000 Da. The unit was centrifuged at 3,000 x g for 30 to 60 min at 4°C. Volumes of concentrates and filtrates were recorded. The bacteriocin activity was assayed for both fractions as described previously. The units were rinsed with 0.1 M NaOH and distilled water and stored with membrane immersed in 0.05 M NaOH.

DE52 Anion Exchange Column Chromatography

Three grams of pre-swollen DE 52 anion exchange cellulose (Whatman, Clifton, NJ) per 10 ml of column volume were added into 0.05 M Tris-HCl buffer (pH 8.0) as recommended by Whatman (1986). While stirring, the slurry was adjusted to pH 8.0 with 1 N HCl. After washing and equilibrating with the buffer 2 to 3 times, the slurry was poured into 2.5 x 20 cm glass column (Bio-Rad) and enough volume of the buffer was eluted until the conductivity measured by conductivity meter (Radiometer, Copenhagen, Denmark) and pH of the eluent were the same as the starting buffer. The final column volume was 50 ml. The flow rate of the eluent was set to 4.0 ml/min by using a pump (Gilson, Middleton, WI) to obtain 48.9 ml/ hr/ cm^2 of the internal cross sectional area.

The concentrated samples from microconcentration were applied to column with 50 ml of 0.05 M Tris-HCl buffer (pH 8), activity was eluted with a linear gradient from 0 to 1.5 M NaCl (200 ml each) in the same buffer. Fractions (4 ml) were collected and measured for absorbance at 280 nm. Each fraction was also assayed for bacteriocin

activity and monitored for conductivity using conductivity meter (Radiometer) to estimate the gradient concentration.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% SDS was carried out by the method of Laemmli (1970) using Mini-Protein slab cell (Bio-Rad, Richmond, CA). The concentrations of polyacrylamide and N'N'-bis-methylene-acrylamide (Bio-Rad) were 12% and 0.32%, respectively in the separating gel, and in stacking gel they were 4% and 0.11%, respectively. The concentration and pH of Tris-HCl buffer were 0.375 M and pH 8.8 in separating gel, and 0.125 M and pH 6.8 in stacking gel. PAGE was conducted at a constant voltage of 200 V for 50 to 60 min. Gels were stained with Coomassie brilliant blue (Bio-Rad). Protein standards and their molecular weight included the following: Lysozyme, 14,400; Carbonic Anhydrase, 31,000; Ovaalbumin, 45,000; bovine serum albumin, 66,200; and phosphorylase B, 97,400 (Bio-Rad).

Protein Determination

The protein content of the fractions was determined by the method of Bradford (1976) using Bio-Rad protein assay reagent. Five ml of diluted dye reagent was added into tubes contain 0.1 ml of samples or distilled water as control. After vortexing gently, the optical density was measured at 595 nm within 1 h. The content of protein was determined by comparing with standard curve which was made using bovine gamma globulin (Bio-Rad).

CHAPTER IV

RESULTS

Inhibitory Action of Spent Broth

A total of 92 strains of *Lactobacillus acidophilus* isolated from fecal contents

of humans, pigs, calves, chickens, rodents, and turkeys were screened for the production

of inhibitory activity (Table 1). Among these strains seventeen out of twenty eight from

pigs, four out of twenty two from calves, three out of nineteen from humans, and three

out of seven from turkeys exhibited inhibitory activity against indicator cultures (*L.*

acidophilus La-1, NCFM-F, and *L. delbrueckii* subsp. *lactis*4797). None of six from

chickens or ten from rodents produced detectable inhibitory actions.

The sensitivity of each strain to inhibitory action also was tested against all of the

strains that produced inhibitory activity against indicator cultures. Among strains that did

not produce inhibitor(s) against the indicator cultures shown in Table 1, the growth of five

from pigs, two from calves, ten from humans, and one from each chicken, rodent, and

turkey were inhibited by some of the inhibitor(s) produced by the twenty seven inhibitory

strains of *L. acidophilus* (Table 2). This shows variations in the inhibitory spectra of the

inhibitor(s) produced by these strains. None of those that produced inhibitory activity

were sensitive to their own inhibitor or inhibitor(s) produced by other strains.

All twenty seven of the inhibitory spent broths were tested for inhibitory action

against *Listeria monocytogenes* Scott A and V7, and *Esherichia coli* 0157:H7 (ATCC

43895). None were inhibitory toward these pathogens. Thus the inhibitory spectrum was

considered to be most likely narrow and active against closely related strains or species.

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TABLE 1

TESTING STRAINS OF *LACTOBACILLUS ACIDOPHILUS* FOR PRODUCTION OF INHIBITORY ACTIVITY

Origin	Test Strains	Indicator Cultures		
		L. acidophilus La-1	*L. acidophilus* NCFM-F	*L. delbrueckii* subsp. *lactis* 4797
Pig	1-3	+A	+	+
	2-5	-	-	-
	107A	+	+	+
	149C	-	-	-
	251	-	-	-
	A1	+	+	+
	A3	+	+	+
	A4	+	+	+
	A6	-	-	-
	C-1-3	-	-	-
	C-1-5	-	-	-
	C-1-6	-	-	-
	C-2-5	-	-	-
	D1	-	-	-
	GP1A	+	+	+
	GP1B	+	+	+
	GP1C	+	+	+
	GP2A	+	+	+
	GP2B	+	+	+
	GP3A	+	+	+
	GP3B	+	+	+
	GP4A	+	+	+
	P16	-	-	-
	P47	+	+	+
	RP32	-	-	-
	RP34	+	+	+
	RP42	+	+	+
	RP43	+	+	+
Calf	25SB	-	-	-
	25SD	-	-	-
	27SC	-	-	-
	30SC	+	+	+
	30SE	-	-	-
	36SC	-	-	-

TABLE 1 (Continued)

Origin	Test Strains	Indicator Cultures		
		L. acidophilus La-1	*L. acidophilus* NCFM-F	*L. delbrueckii* subsp. *lactis* 4797
Calf	381-DUO-20	+	+	+
	381-IL-23	-	-	-
	381-IL-25	-	-	-
	381-IL-27	-	-	-
	381-IL-28	+	+	+
	396-IL-28	-	-	-
	C28	-	-	-
	FR-1	-	-	-
	FR-2	+	+	+
	FR-3	-	-	-
	FR-4	-	-	-
	FR-5	-	-	-
	FR-6	-	-	-
	R-1	-	-	-
	R-2	-	-	-
	R-3	-	-	-
Human	107	-	-	-
	223	-	-	+
	606	-	-	+
	4356	-	-	-
	4962	-	-	-
	HM2	-	-	-
	H35	-	-	-
	La 1	-	-	-
	La 2	-	-	-
	La 3	-	-	-
	La 5	-	-	-
	La 8	-	-	-
	La 11	-	-	-
	La 12	-	-	-
	La 14	-	-	-
	La 15	-	-	-
	La 20	-	-	-
	NCFM-L	-	-	-
	NCFM-M	+	+	+
	NCFM-F	-	-	-

TABLE 1 (Continued)

Origin	Test Strains	Indicator Cultures		
		L. acidophilus La-1	*L. acidophilus* NCFM-F	*L. delbrueckii* subsp. *lactis* 4797
Chicken	5L3	-	-	-
	5S3	-	-	-
	6L4	-	-	-
	6S3	-	-	-
	6S4	-	-	-
	8L3	-	-	-
Rodent	NFa-3	-	-	-
	NFa-4	-	-	-
	NFa-5	-	-	-
	NFa-8	-	-	-
	PLa-9	-	-	-
	PLb-3	-	-	-
	PLb-5	-	-	-
	PLb-6	-	-	-
	PLb-10	-	-	-
	Rat-1	-	-	-
Turkey	T1	-	-	-
	T2	+	+	+
	T3	+	+	+
	T4	-	-	-
	A1-Turkey	+	+	+
	A2-Turkey	-	-	-
	C2-Turkey	-	-	-

A + indicates the presence of zone of inhibition; - indicates the absence of zone of inhibition on the agar seeded with each indicator cultures

TABLE 2

COMPARISON OF SENSITIVITY TO THE INHIBITORY ACTIVITY PRODUCED
BY STRAUINS OF *LACTOBACILLUS ACIDOPHILUS*

Origin	Producer Strains	Nonproducer Strains from Pigs				
		149C	C-1-3	C-1-5	C-1-6	C-2-5
Pig	1-3	+A	+	+	+	-
	107A	+	+	+	+	+
	A1	+	-	+	+	+
	A3	+	-	+	+	+
	A4	+	+	+	+	+
	GP1A	-	-	-	-	+
	GP1B	+	+	+	+	-
	GP1C	+	+	+	+	+
	GP2A	-	+	+	+	+
	GP2B	-	+	+	+	+
	GP3A	+	+	+	+	-
	GP3B	+	+	+	+	+
	GP4A	+	+	+	+	-
	P47	+	+	+	+	-
	RP34	+	+	+	+	+
	RP42	-	+	+	+	+
	RP43	+	+	+	+	+
Calf	30SC	+	+	+	+	+
	381-DUO-20	-	-	-	-	-
	381-IL-28	+	-	-	-	-
	FR-2	-	+	+	+	+
Human	223	NDB	-	-	-	-
	606	ND	-	-	-	-
	NCFM-M	ND	+	+	+	+
Turkey	T2	-	ND	ND	ND	-
	T3	-	ND	ND	ND	-
	A1-Turkey	+	ND	ND	ND	-

TABLE 2 (Continued)

Origin	Producer Strains	Nonproducer Strains from Humans				
		La-1	La-2	La-5	La-8	La-11
Pig	1-3	+	+	+	+	+
	107A	+	+	+	+	+
	A1	+	+	+	+	+
	A3	+	+	+	+	+
	A4	+	+	+	+	+
	GP1A	+	+	+	+	+
	GP1B	+	+	+	+	+
	GP1C	+	+	+	+	+
	GP2A	+	+	+	+	+
	GP2B	+	+	+	+	+
	GP3A	+	+	+	+	+
	GP3B	+	+	+	+	+
	GP4A	+	+	+	+	+
	P47	+	+	+	+	+
	RP34	+	+	+	+	+
	RP42	+	+	+	+	+
	RP43	+	+	+	+	+
Calf	30SC	+	+	+	+	+
	381-DUO-20	+	+	-	-	-
	381-IL-28	+	+	+	+	+
	FR-2	+	+	-	-	-
Human	223	-	-	-	-	-
	606	-	-	+	+	+
	NCFM-M	+	+	+	+	+
Turkey	T2	+	+	+	+	+
	T3	+	+	+	+	+
	A1-Turkey	+	+	+	+	+

TABLE 2 (Continued)

Origin	Producer Strains	Nonproducer Strains from Humans				
		La-12	La-14	La-15	La-20	NCFM-F
Pig	1-3	+	+	+	+	+
	107A	+	+	+	+	+
	A1	+	+	+	+	+
	A3	+	+	+	+	+
	A4	+	+	+	+	+
	GP1A	+	+	+	+	+
	GP1B	+	+	+	+	+
	GP1C	+	+	+	+	+
	GP2A	+	+	+	+	+
	GP2B	+	+	+	+	+
	GP3A	+	+	+	+	+
	GP3B	+	+	+	+	+
	GP4A	+	+	+	+	+
	P47	+	+	+	+	+
	RP34	+	+	+	+	+
	RP42	+	+	+	+	+
	RP43	+	+	+	+	+
Calf	30SC	+	+	+	+	+
	381-DUO-20	-	-	-	-	+
	381-IL-28	-	-	-	-	+
	FR-2	-	-	-	-	+
Human	223	-	-	+	+	-
	606	-	+	+	+	-
	NCFM-M	+	+	+	+	+
Turkey	T2	ND	ND	ND	ND	+
	T3	ND	ND	ND	ND	+
	A1-Turkey	ND	ND	ND	ND	+

TABLE 2 (Continued)

Origin	Producer Strains	Nonproducer Strains			
		Calf		Chicken	Rodent
		149C	C-1-3	C-1-5	C-1-6
Pig	1-3	-	-	-	+
	107A	+	+	+	+
	A1	-	+	-	-
	A3	+	+	+	+
	A4	-	-	-	-
	GP1A	-	-	+	-
	GP1B	-	-	+	-
	GP1C	-	+	-	+
	GP2A	-	-	+	-
	GP2B	-	-	+	-
	GP3A	-	-	+	-
	GP3B	-	-	+	-
	GP4A	-	-	+	-
	P47	-	-	-	-
	RP34	-	-	+	-
	RP42	+	+	-	-
	RP43	+	+	-	-
Calf	30SC	-	-	+	-
	381-DUO-20	-	-	-	-
	381-IL-28	-	-	-	+
	FR-2	-	-	+	-
Human	223	-	-	-	-
	606	-	-	+	-
	NCFM-M	+	+	+	+
Turkey	T2	+	-	+	-
	T3	+	-	+	-
	A1-Turkey	-	-	+	+

A + indicates the presence of zone of inhibition; - indicates the absence of zone of inhibition on the agar seeded with each indicator cultures

B Not determined

Effects of Catalase and Protease on Inhibition

The cell-free spent broths of strains that produced inhibitory action were treated with the proteolytic enzymes pepsin and trypsin. The inhibitory substance for each strain was completely inactivated by treatment with either of these proteolytic enzymes (Table 3). The results indicated that the inhibitory substances were proteinous compounds.

In addition, the spent broths were treated with catalase to eliminate any inhibitory effect of hydrogen peroxide. Inhibitory activity of the spent broths was not affected by catalase treatments (Table 3).

Effects of Heat on Inhibitory Activity

The inhibitory spent broths were heated at 121°C for 15 min, and assayed for inhibitory activity (Table 4). No differences were observed in the formation of inhibitory zones on the produced by twenty seven strains of *Lactobacillus acidophilus* were heat stable proteinous compounds. Neither hydrogen peroxide nor acid were responsible for inhibitory activity against indicator cultures. Antagonism by these inhibitors was restricted agar plates seeded with indicator strains between heated and unheated spent broth indicating that the inhibitory materials were heat stable.

Purification of Bacteriocins

Results from the above experiments demonstrated that the inhibitory substance(s) to closely related species. None of the strains that produced inhibitory substances was sensitive to its own inhibitors or inhibitor(s) produced by other strains. The inhibitory activity of spent broth was non-dialyzable in dialysis membrane tubing with molecular weight exclusion limits of 12 to 14 KDa. The activity was also retained when concentrated by microconcentrator with molecular weight limit of 30 KDa. These results show the

TABLE 3

EFFECTS OF CATALASE AND PROTEASE ON INHIBITORY ACTIVITY
PRODUCED BY STRAINS OF *LACTOBACILLUS ACIDOPHILUS*

Origin	Test Strains	Enzyme Treatment			
		Control	Catalase	Trypsin	Pepsin
Pig	1-3	+A	+	-	-
	107A	+	+	-	-
	A1	+	+	-	-
	A3	+	+	-	-
	A4	+	+	-	-
	GP1A	+	+	-	-
	GP1B	+	+	-	-
	GP1C	+	+	-	-
	GP2A	+	+	-	-
	GP2B	+	+	-	-
	GP3A	+	+	-	-
	GP3B	+	+	-	-
	GP4A	+	+	-	-
	P47	+	+	-	-
	RP34	+	+	-	-
	RP42	+	+	-	-
	RP43	+	+	-	-
Calf	30SC	+	+	-	-
	381-DUO-20	+	+	-	-
	381-IL-28	+	+	-	-
	FR-2	+	+	-	-
Human	223	+	+	-	-
	606	+	+	-	-
	NCFM-M	+	+	-	-
Turkey	T2	+	+	-	-
	T3	+	+	-	-
	A1-Turkey	+	+	-	-

A + indicates the presence of zone of inhibition; - indicates the absence of zone of inhibition on the agar seeded with *L. delbrueckii* subsp. *lactis* 4797

TABLE 4

EFFECTS OF HEAT ON INHIBITORY ACTIVITY PRODUCED BY STRAINS OF *LACTOBACILLUS ACIDOPHILUS*

Origin	Test Strains	Unheated	Heated
Pig	1-3	$-$A	$-$
	107A	$+$	$+$
	A1	$+$	$+$
	A3	$+$	$+$
	A4	$+$	$+$
	GP1A	$+$	$+$
	GP1B	$+$	$+$
	GP1C	$+$	$+$
	GP2A	$+$	$+$
	GP2B	$+$	$+$
	GP3A	$+$	$+$
	GP3B	$+$	$+$
	GP4A	$+$	$+$
	P47	$+$	$+$
	RP34	$+$	$+$
	RP42	$+$	$+$
	RP43	$+$	$+$
Calf	30SC	$+$	$+$
	381-DUO-20	$+$	$+$
	381-IL-28	$+$	$+$
	FR-2	$+$	$+$
Human	223	$+$	$+$
	606	$+$	$+$
	NCFM-M	$+$	$+$
Turkey	T2	$+$	$+$
	T3	$+$	$+$
	A1-Turkey	$+$	$+$

A $+$ indicates the presence of zone of inhibition; $-$ indicates the absence of zone of inhibition on the agar seeded with *L. delbrueckii* subsp. *lactis* 4797.

inhibitor(s) to be consistent to the definition of bacteriocins as defined by Tagg et. al. (1976).

Based on consistency in production of inhibitory activity, and identity characteristics (e.g. strain identification according to Buchanan and Gibbson, 1974) throughout the study, fourteen of the twenty seven inhibitory strains were selected for further study. Attempts to purify bacteriocins produced by them involved ammonium sulfate precipitation and Sephadex G-200 gel chromatography. The elution profiles are presented in Figures 5 through 17 in Appendix B. The bacteriocins produced by some strains lost their activity relatively faster than others after this series of purification steps. Bacteriocins produced by eight of the strains were stable through purification steps, and subjected to further purification. The eight strains included of one from human origin (*L. acidophilus* 606), two strains from calves (*L. acidophilus* 30SC and FR-2), and five strains from pigs (*L. acidophilus* A4, GP1B, GP2A, GP4A, and RP42).

Ammonium Sulfate Precipitation

The results of purification of bacteriocins produced by eight strains of *L. acidophilus* are given in Tables 7 through 14 in Appendix A. The specific activity was calculated as total activity (AU) of inhibitory activity per total protein (mg) in each fraction. The comparison of specific activities and degrees of purification of the eight strains of *L. acidophilus* at each purification step are shown in Table 5. A 50% ammonium sulfate precipitation resulted in 6.3 (*L. acidophilus* GP4A), 9.8 (*L. acidophilus* 30SC), 18.0 (*L. acidophilus* GP2A), 19.3 (*L. acidophilus* GP1B), 19.6 (*L. acidophilus* RP42), 20.7 (*L. acidophilus* A4), 27.8 (*L. acidophilus* FR2), and 48.6 (*L. acidophilus* 606) fold increases in specific activities compared to spent broth.

Total activity (AU) of the fractions at each purification step was divided by total activity of spent broth to obtain the percentage of total activity recovered at each step (Tables 8 through 15 in Appendix A). The comparison of the percentage of bacteriocin

TABLE 5

COMPARISON OF SPECIFIC ACTIVITY[A] OF BACTERIOCIN FROM EACH
STRAINS OF *LACTOBACILLUS ACIDOPHILUS*

Strain	Spent Broth	$(NH4)_2SO_4$[B] Precipitation	Gel Filtration	Microconcentrate
606	2,018.9	98,107.8 (48.6)[C]	78,056.2 (38.7)	330,876.0 (163.9)
30SC	627.5	6,159.4 (9.8)	14,209.9 (22.6)	81,835.5 (130.5)
FR2	39.2	1,089.4 (27.8)	797.4 (20.3)	6,154.8 (156.9)
A4	2,509.8	52,012.7 (20.7)	46,400.5 (18.5)	260,076.4 (103.6)
GP1B	336.8	6,501.6 (19.3)	5,404.2 (16.0)	42,010.3 (124.7)
GP2A	4,249.0	76,650.3 (18.0)	54,677.1 (12.9)	779,610.4 (183.5)
GP4A	11,058.3	70,204.6 (6.3)	65,878.6 (6.0)	509,017.5 (46.0)
RP42	42.1	825.8 (19.6)	808.6 (19.2)	4,776.7 (113.4)

[A] Total Activity (AU) per Total Protein (mg)
[B] At 50% saturation
[C] Values in Parentheses Indicate the Fold Purification

TABLE 6

COMPARISON OF % OF TOTAL BACTERIOCIN ACTIVITY RECOVERED[A] AT
EACH PURIFICATION STEP FROM STRAINS OF *LACTOBACILLUS
ACIDOPHILUS*

Strain	Spent Broth	$(NH4)_2SO_4$[B] Precipitation	Gel Filtration	Microconcentrate
606	100.0	93.9	72.5	68.3
30SC	100.0	76.5	69.6	55.7
FR2	100.0	89.6	72.5	68.3
A4	100.0	70.7	60.6	53.9
GP1B	100.0	81.9	67.0	59.5
GP2A	100.0	83.0	69.2	66.4
GP4A	100.0	83.5	69.6	55.7
RP42	100.0	80.8	67.4	53.9

[A] Total Activity (AU) of Each Fraction per Total Activity (AU) of Spent Broth
[B] At 50% saturation

activity recovered among the eight strains is presented in Table 6. A 70.7%(*L. acidophilus* A4) to a 93.9% (*L. acidophilus* 606) of activity were recovered by ammonium sulfate precipitation.

The ammonium sulfate precipitation eliminated 86.9% (*L. acidophilus* GP4A), 92.2% (*L. acidophilus* 30SC), 95.4% (*L. acidophilus* GP2A), 95.8% (*L. acidophilus* GP1B), 95.9% (*L. acidophilus* RP42), 96.6% (*L. acidophilus* A4), 96.8% (*L. acidophilus* FR2), and 98.1% (*L. acidophilus* 606) of protein in the spent broths.

Sephadex G-200 Gel Column Chromatography

The resolved portions of ammonium sulfate precipitate from spent broths of strains of *L. acidophilus* were applied to a Sephadex G-200 gel column and eluted with 0.05 M Tris buffer (pH 8.0). The eluent was collected in 5 ml fractions and the absorbance 280 nm and the inhibitory activity were measured. The inhibitory activity was eluted as the void volume between two absorbance peaks of contaminating proteins as Figure 1 which shows the elution profile of *L. acidophilus* GP4A. Other strains also demonstrated similar elution pattern as *L. acidophilus* GP4A (Data not shown).

Gel chromatography of the ammonium sulfate precipitate fraction of spent broth from *L. acidophilus* GP4A in the presence of 0.1% SDS resulted in the elution of a single peak of inhibitory activity corresponding to a single absorbance peak (Fig. 2). In addition, larger portion of material absorbing light at 280 nm was fractionated as a second peak in which no inhibitory activity was detected. The inhibitory activity of thirteen other strains of *L. acidophilus* from pigs, three strains from cows, and one strain from human origin were subjected to gel chromatography in the presence of 0.1% SDS. As shown in Figures 5 through 17 in Appendix B, the elution profiles of all strains tested were similar to that of *L. acidophilus* GP4A. The active fraction was coeluted with the first absorbance peak which was eluted right after the void volume. Other material that absorbed light at 280 nm
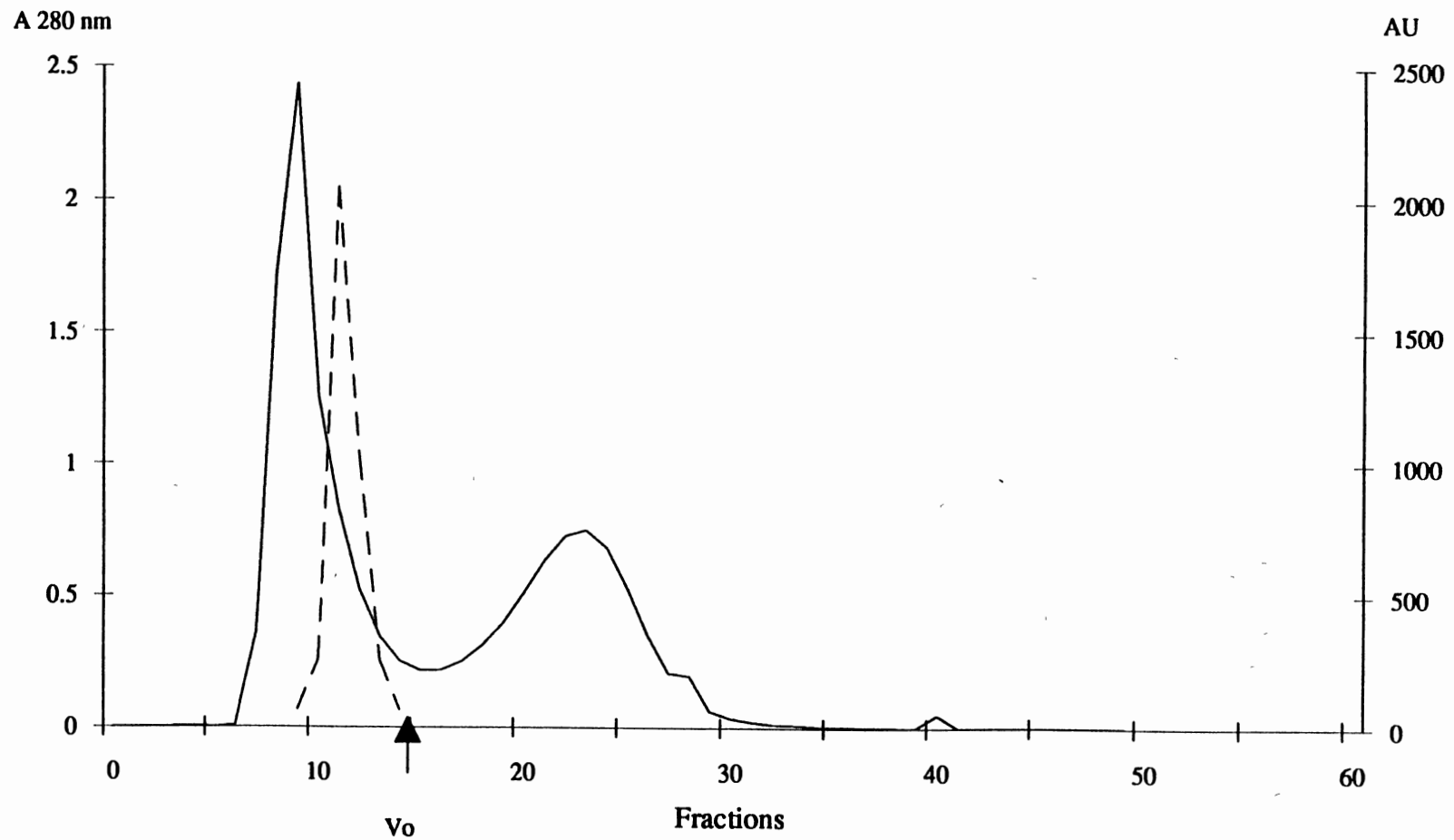
Figure 1. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* GP4A on Sephadex G-200 without SDS. Each 5 ml fraction was monitored at 280 nm (———) and was assayed for inhibitory activity (- - - -).
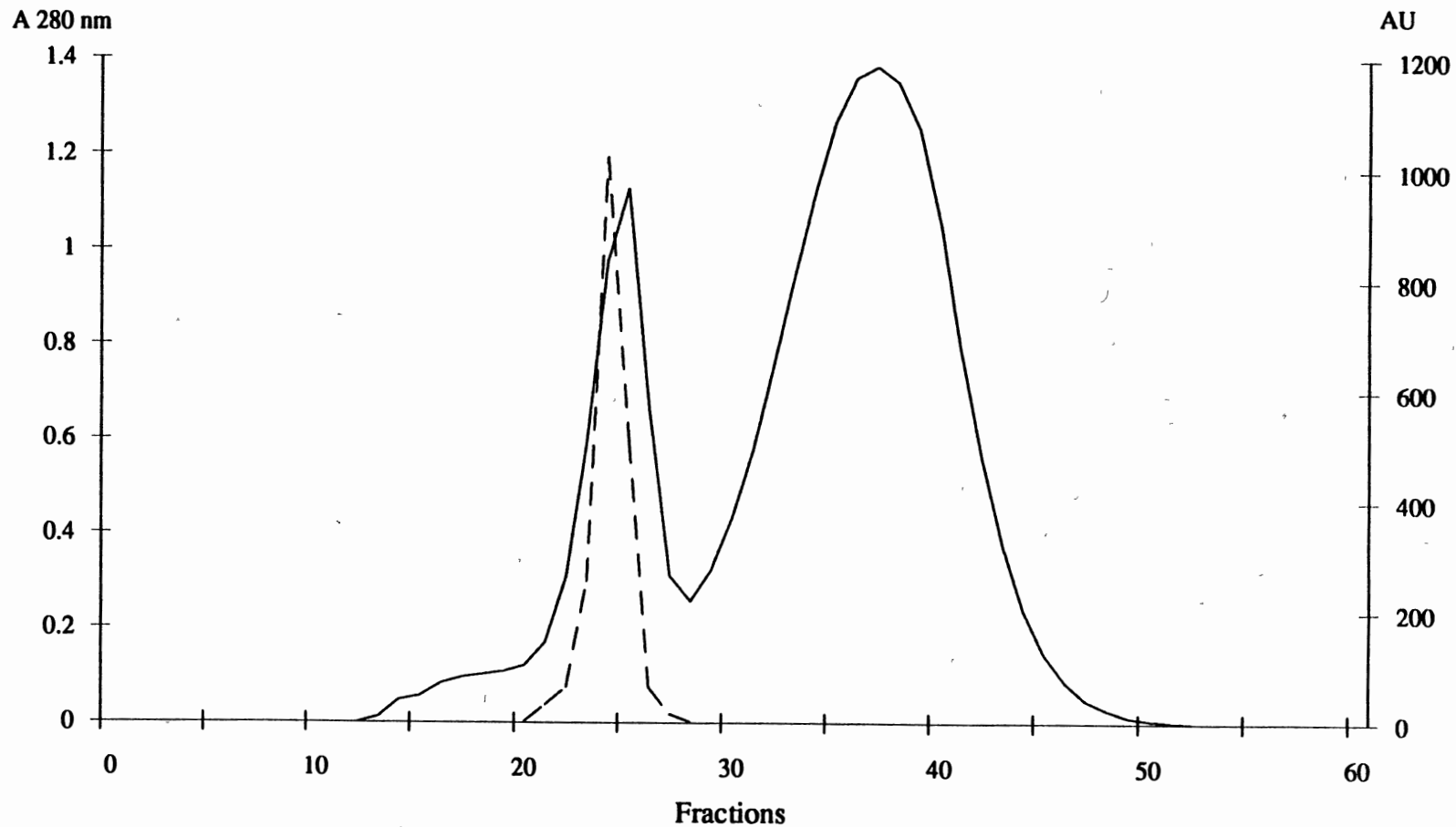
Figure 2. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* GP4A on Sephadex G-200 in the presence of 0.1% SDS. Each 5 ml fraction was monitored at 280 nm (———) and was assayed for inhibitory activity (- - - -).

appeared in the second peak. The highest points in absorbance and activity were not always eluted as a same fraction.

Table 5 shows that a 6.0 fold (*L. acidophilus* GP4A) to 38.7 fold (*L. acidophilus* 606) purifications compared to the original spent broths were achieved by gel chromatography. The amounts of activity recovered were from 60.6% (*L. acidophilus* A4) to 72.5% (*L. acidophilus* FR2) of original activity in cell free spent broth (Table 6).

The active fractions from gel chromatography were pooled and concentrated with Centricell microconcentrator. The resulting concentrated solutions yield a 46 (*L. acidophilus* GP4A) to 163.9 (*L. acidophilus* 606) fold purification (Table 5). Table 6 shows percentages of original inhibitory activity (spent broth) recovered ranged from 53.9% (*L. acidophilus* A4 and RP42) to 68.3% (*L. acidophilus* 606 and FR2).

Ion Exchange Chromatography

The inhibitory fraction from *L. acidophilus* GP4A obtained by gel filtration was microconcentrated and subjected to anion exchange column chromatography. Two absorbance peaks at 280 nm were detected. First peak was eluted before the sodium chloride gradient was applied. The inhibitory activity was eluted with second absorbance peak at A 280nm (Fig. 3). The conductivity of the active fraction was between 20 to 30 ms which corresponded to 0.4 M sodium chloride. Diffusion of very weak activity throughout most fractions was observed. The elution profiles of the seven other strains of *L. acidophilus* on ion exchange chromatography are presented in Figures 18 through 24 in Appendix C. All of strains exhibited similar elution patterns. The bacteriocin activity was coeluted with second absorbance peak which was at about 0.4 M NaCl. Diffusion of activity throughout the fractions also was observed for all strains.

The ion exchange chromatography resulted in 15.7 (*L. acidophilus* 606) to 2.0 (*L. acidophilus* GP4A) fold increases in specific activity. The amounts of bacteriocin activity
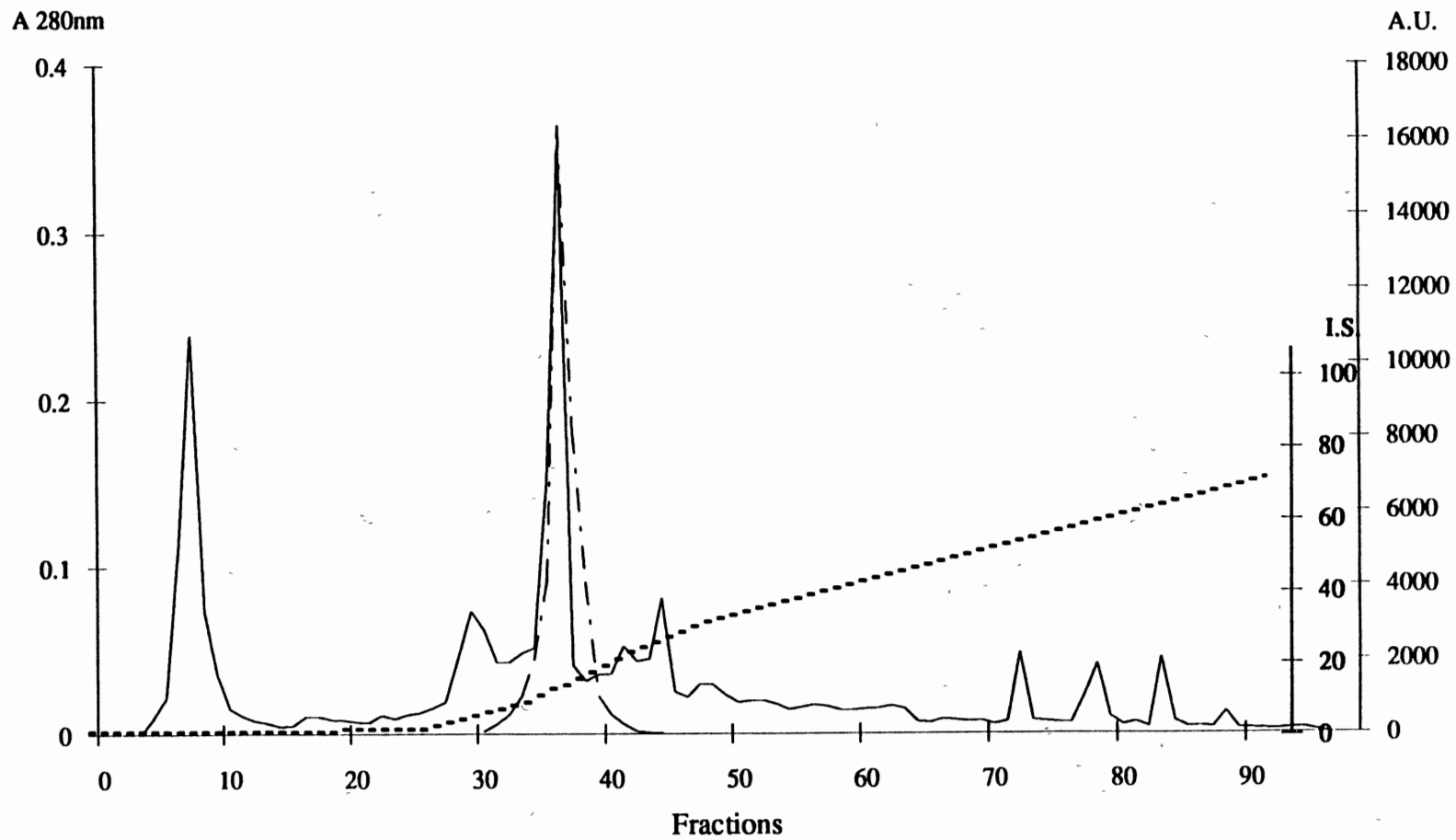
Figure 3. Chromatography of inhibitory activity obtained from Sephadex G-200 produced by *L. acidophilus* GP4A on DE 52 anion exchange. The activity was eluted with 0 to 1.5 M NaCl. Each 4 ml fraction was monitored for absorbance at 280 nm (————) and for conductivity (-----), and was assayed for inhibitory activity (— – —).

recovered were from 16.8% (*L. acidophilus* A4 and RP42) to 34.6% (*L. acidophilus* GP2A) of activity in the spent broths (Tables 7 through 14).

SDS Polyacrylamide Gel Electrophoresis

The active fractions from the second peak of ion exchange columns were electrophoresed. As demonstrated in Figure 4, 72 KDa bands appeared in common in the active fractions from all eight strains of *L. acidophilus*.

Attempts to show inhibition zone on the indicator lawn of agar by the bands from gels were not successful, possibly because of lack of sufficient inhibitory substances to produce inhibitory zone on the agar.

72 KDa →

1   2   3   4   5   6   7 |8   9
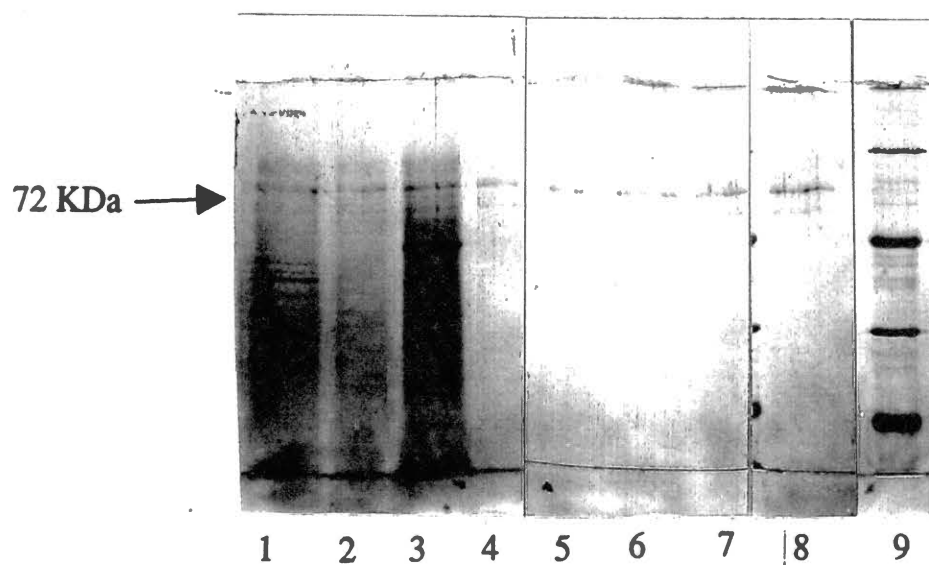
Figure 4. Coomassie blue stained SDS-PAGE gel of inhibitory activity
fractions from DE 52 ion exchange. Lane 1, *L. acidophilus*
GP1B; Lane 2, *L. acidophilus* GP2A; Lane 3, *L. acidophilus*
GP4A; Lane 4, RP42, Lane 5, *L. acidophilus* A4; Lane 6, *L.*
acidopilus 30SC; Lane 7, *L.acidophilus* FR-2; Lane 8, *L.*
acidophilus 606; Lane 9,protein standard.

CHAPTER V

DISCUSSION

Bacteriocins are protein or protein related complexes with inhibitory action directed against species that are usually closely related to the producer bacterium (Tagg et. al., 1976). Since Rogers (1928) first indicated the presence of antibiotics among lactic acid bacteria, a number of lactic acid bacteria including strains of *L. acidophilus* (Barefoot and Klaenhammer, 1984; Muriana and Klaenhammer, 1991a), *L. helveticus* (Upreti and Hinsdill, 1975; Joerger and Klaenhammer, 1986), *L. fermenti* (De Klerk and Smit, 1967), *L. plantarum* (Daeschel et. al., 1986;West and Warner, 1988; Anderson et. al., 1988), *L. sake* (Schillinger and Luke, 1989; Mortvedt and Nes, 1990), and *L. casei* (Rammelsberg et. al., 1990) have been shown to produce bacteriocins or bacteriocin-like inhibitory substances.

Twenty seven strains among 92 strains of *L. acidophilus* isolated from fecal contents of humans, pigs, calves, rodents , and turkeys demonstrated inhibitory activity against bacteriocin sensitive strains including *L. acidophilus* La-1, NCFM-F, and *L. delbrueckii* subsp. *lactis* 4797. No strain that produced inhibitory activity was sensitive to its own bacteriocin or bacteriocins produced by other strains. These results fit the description of bacteriocin in that the bacteriocin-producing strain has host cell immunity (Tagg et. al. 1976).

The inhibitory activities produced by the strains of *L. acidophilus* in this study were not effective against strains of *Listeria monocytogenes* and *E. coli*. Ferreira and Gilliland (1988) and other researchers (Barefoot and Klaenhammer, 1983; Muriana and Klaenhammer, 1987) showed limited inhibitory activity of bacteriocins produced by

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strains of *L. acidophilus* against closely related species. Tagg et. al. (1976) defined a narrow inhibitory spectrum as one characteristic of a bacteriocin. Although some cultures of lactobacilli (Schillinger and Luke, 1989; McCormick and Savage, 1983) and *Pediococci* species (Hoover et. al., 1988; Bhunia et. al., 1988; Pucci et. al., 1988; Spelhaug and Harlander, 1989; Nielsen et. al., 1990; Yousef et. al., 1991) exhibited the antagonistic activity against *Listeria monocytogenes* and/or other forborne pathogens, only lactacin F (Muriana and Klaenhammer, 1991a) has been reported to be active against some pathogens among confirmed bacteriocins produced by strains of the species *L. acidophilus*.

All inhibitory activity produced by twenty seven strains of *L. acidophilus* were sensitive to the proteolytic enzymes pepsin and trypsin, heat stable at $121^{\circ}C$ for 15 min, and resistant to destruction by catalase. These results meet the criteria described by Tagg et. al. (1976) of bacteriocins from Gram-positive bacteria and confirm the identity of inhibitory materials produced by the cultures of *L. acidophilus* in the present study as bacteriocins.

Vincent et. al. (1959) tried to purify a bacteriocin (lactocidin) produced by *L. acidophilus*. Since then many attempts have been made to purify bacteriocins from *L. acidophilus* and other lactobacilli (Barefoot and Klaenhammer, 1983 and 1984; Muriana and Klaenhammer, 1991a; Mortvedt et. al., 1991). Because the bacteriocins are proteinous compounds, several researchers have employed ammonium sulfate precipitation as their initial purification step. A 60% saturation of spent media with ammonium sulfate solution was used to precipitate the diplococcin (Davey and Richardson, 1981), propionicin PLG-1 (Lyon and Glatz, 1991), and lactacin 481 (Piard et. al., 1992). Helveticin J, a bacteriocin produced by *L. helveticus* 481, was precipitated by 50% saturation with ammonium sulfate (Joerger and Klaenhammer, 1986). Thirty five to forty % and twenty % saturation with ammonium sulfate were employed to purify lactacin F (Muriana and Klaenhammer, 1991a) and lactocin S (Mortvedt et. al., 1991), respectively. The majority of the inhibitory

activity was recovered while a great portion of contaminating protein was eliminated through the ammonium sulfate precipitation (Mortvedt et. al.,1991; Muriana and Klaenhammer, 1991a; Piard et. al., 1992). In the present study, the bacteriocin(s) produced by strains of *L. acidophilus* was precipitated at 50% ammonium sulfate saturation. More than 70.7% of bacteriocin activity of original spent broth was recovered in the precipitate while more than 86.9% of protein from spent broth was eliminated.

The inhibitory fraction recovered by ammonium sulfate precipitation of spent broth from *L. acidophilus* GP4A was subjected to gel filtration chromatography . The bacteriocin activity was eluted in the void volume in the absence of SDS. However, when the same fraction was eluted from the gel column with buffer containing 0.1% SDS, the activity was included in a peak corresponding to a single absorbance peak which eluted after the void volume. The detergent SDS denatures protein by association of the apolar tails of the SDS molecule with protein hydrophobic groups (Zubay, 1988). This denaturation resulted in dissociation of large aggregate of subunits of a protein. The results from the gel chromatographic purification of the bacteriocin in this study indicated that the bacteriocin was present as an aggregate and dissociated to smaller subunits when exposed to SDS. The presence of large molecular complex of bacteriocins produced by lactobacilli has been reported including lactacin B (Barefoot and Klaenhammer, 1983 and 1984), lactocin 27 (Upreti and Hinsdill, 1973), helveticin J (Joerger and Klaenhammer, 1986), and lactacin F (Muriana and Klaenhammer, 1991a). Lyon and Glatz (1991) also observed that propionicin PLG-1 produced by *Propionibacterium thoenii* was present as two protein aggregates. Interestingly, the elution profiles of lactocin 27 produced by *L. helveticus* LP27 (Upreti and Hinsdill, 1973) on gel chromatography was very similar to those observed in the present study when eluted with or without SDS.

The active fractions collected from gel chromatography were concentrated and applied to ion exchange chromatography for further purification. In preliminary experiments, the bacteriocin activity was absorbed to anion exchange resin at pH 8.0 and

eluted with sodium chloride gradient whereas the inhibitory activity was not absorbed to the cation exchange material. Anion exchange chromatography yielded two distinctive absorbance peaks (280 nm). Only one of these two fractions contained the inhibitory activity. However, a great portion of the bacteriocin activity was lost during this step. In addition, very weak inhibitory activity appeared throughout the fractions from ion exchange chromatography. Difficulties have been observed using ion exchange chromatography to purify some bacteriocins (Ferreira, 1986). Possible reasons suggested for the difficulties include either too strong absorption of bacteriocin to charged surfaces (Ellison and Kauter, 1970; Tagg and Russel, 1981), or denaturation of protein during passage through the ion exchange column (Clark and Switzer, 1977). The strong tendency of bacteriocins to be absorbed to the charged surfaces may have attributed to loss and diffusion of activity. Barefoot and Klaenhammer (1984) also attempted ion exchange chromatography to purify lactacin B produced by *L. acidophilus* N2. However, a majority of the activity was lost during the process. They suggested that lactacin B had either been denatured or was not soluble in the acetate buffer system at high concentration. Caseicin 80 produced by *L. casei* B80 was purified by cation exchange chromatography (up to 68 fold increase in specific activity) but 50 to 80% of the activity was lost (Rammelsberg et. al., 1990). However, other researchers have successfully purified bacteriocins by ion exchange chromatography including acidophilin (Shahani et. al., 1977), acidolin (Mikolajcik and Hamdan, 1975), and lactocin S (Mortvedt et. al., 1991).

The active fractions from strains of *L. acidophilus* eluted from ion exchange chromatography were microconcentrated and applied on SDS-PAGE. A 72 KDa band commonly appeared for all eight strains of *L. acidophilus*. Since this band was only one common in the inhibitory fractions from the 8 strains tested, it appears that it was responsible for the bacteriocin activity of all eight strains. Many different molecular weights of bacteriocins have been reported which include: 1.7 KDa for lactacin 481 (Piard et. al., 1992), 2.5 KDa for lactacin F (Muriana and Klaenhammer, 1991a), 2.7 KDa for

pediocin AcH (Bhunia et. al.), 4.5 KDa for mesentrocin 5 (Daba et. al., 1991), 5.4 KDa for a bacteriocin from *L. acidophilus* AC_1, 6 to 6.5 KDa for lactacin B (Barefoot and Klaenhammer, 1984), 10 KDa for propionicin PLG-1 (Lyon and Glatz, 1991), 12.4 KDa for lactocin 27 (Upreti and Hinsdill, 1973)16.5 KDa for pediocin PA-1 (Gonzalez and Kunka, 1987), 37 KDa for helveticin J (Joerger and Klaenhammer, 1986), and 40 to 42 KDa for caseicin 80 (Rammelsberg et. al., 1990).

However, true comparisons of molecular weights and sizes of these bacteriocins still remains questionable. First, most of researchers employed different methods to prepare the samples and determined molecular weight under different conditions. Second, bacteriocins could easily be associated with other molecules or subunits to form complex compound (Muriana and Klaenhammer, 1991; Upreti and Hinsdill, 1973). Third, even when SDS-PAGE had been used under similar condition to estimate the molecular weight, factors other than size of the protein can affect the migration of protein on electrophoretic field and result in incorrect molecular weight estimations especially if the large native protein is present (Hames and Rickwood, 1981). In addition, Piard et. al. (1992) and Holo et. al. (1991) observed discrepancy in estimating molecular weight by SDS-PAGE or by amino acid composition analysis.

Although there are differences in characteristics and properties of reported bacteriocins produced by lactobacilli, the results of this study indicated that the bacteriocins produced by eight strains of *L. acidophilus* had similar characteristics. Further studies including amino acid sequence analyses and genetic determination of bacteriocin production and host immunity are needed to confirm whether there are differences in bacteriocins produced by various strains of *L. acidophilus*.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Twenty seven strains among 92 strains of *Lactobacillus acidophilus* isolated from fecal contents of humans, pigs, calves, rodents, and turkeys demonstrated inhibitory activity against bacteriocin sensitive strains including *L. acidophilus* La-1, NCFM-F, and *L. delbrueckii* subsp. *lactis* 4797. The inhibitory substance(s) produced by these strains of *L. acidophilus* were heat stable and non-dialyzable proteinous compounds. Neither hydrogen peroxide nor acid were responsible for inhibitory actions. The inhibitor(s) exhibited narrow inhibitory spectra of activity. All of the producer strains were resistant to their own inhibitor or inhibitor(s) produced by other strains. The observed characteristics fit the description of bacteriocins produced by Gram-positive bacteria (Tagg et. al., 1976) so that the inhibitory substance(s) produced by 27 strains of *L. acidophilus* was classified as bacteriocin(s).

The bacteriocin(s) was purified by ammonium sulfate precipitation at 50% saturation. More than 70.7% of bacteriocin activity was recovered in the precipitate while more than 86.9% of protein from spent broth was eliminated.

Different patterns of elution were obtained when the ammonium sulfate precipitate from spent broth was applied to a Sephadex G-200 gel column and eluted with buffer with or without SDS. This result might indicated the presence of large aggregate of a protein. The elution profiles of all strains tested on a Sephadex G-200 column resulted in two absorbance peaks. The active fraction was coeluted as first peak right after void volume. As much as 163.9 fold purification and 68.3% recovery of amount of activity were

achieved by gel column chromatography when the active fractions were concentrated with microconcentrator.

For further purification of bacteriocin, DE-52 anion exchange column chromatography was employed. The bacteriocin activity was coeluted with second absorbance peak which was eluted at 0.4 M NaCl. There were some loss of activity and no improvement in degree of purification by the ion exchange chromatography. SDS-PAGE of the active fractions from ion exchange chromatography revealed a 72 KDa band which was common for all eight strains of *L. acidophilus* tested. Because of the presence of only one band in common, it appears that this band was responsible for the bacteriocin activity of all strains tested.

Great diversity in characteristic and properties of bacteriocins produced by lactobacilli have been reported while they meet most of criteria set by Tagg et. al. (1976). In this study, however, the results indicated that the bacteriocin(s) produced by strains of *L. acidophilus* isolated from different origins exhibited similar characteristics.

Future studies should include amino acid sequence analysis and genetic determination of bacteriocin production and host immunity to determine whether there are differences in bacteriocins produced by various strains of *L. acidophilus*.

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APPENDIX A

PURIFICATION OF BACTERIOCIN PRODUCED BY

EIGHT STRAINS OF *LACTOBACILLUS ACIDOPHILUS*

TABLE 7

PURIFICATION OF BACTERIOCIN PRODUCED BY *LACTOBACILLUS ACIDOPHILUS* 606

	Spent Broth	Precipitate	Gel Filtration	Micro-concentrate	Ion exchange
Volume	3,000.0	11.0	17.0	0.5	5.0
Protein Concn.(mg/ml)	0.13	0.67	0.42	3.17	0.10
Total Protein(mg)	380.40	7.35	7.14	1.58	0.52
Bacteriocin Activity (AU/ml)	256	65,536	32,768	1,048,579	16,384
Total Activity(AU)	768,000	720,896	557,056	524,290	81,920
Specific Activity (AU/mg)	2,018	98,107	78,056	330,876	158,207
Activity Recovered (%)	100.0	93.9	72.5	68.3	10.7
Purification (fold)	1.0	48.6	38.7	163.9	78.4

TABLE 8

PURIFICATION OF BACTERIOCIN PRODUCED BY *LACTOBACILLUS ACIDOPHILUS* 30SC

	Spent Broth	Precipitate	Gel Filtration	Micro-concentrate	Ion exchange
Volume	1,840.0	11.0	20.0	0.5	5.0
Protein Concn.(mg/ml)	0.20	2.66	0.58	3.20	0.17
Total Protein(mg)	375.4	29.3	11.5	1.6	0.84
Bacteriocin Activity (AU/ml)	128	16,384	8,192	262,144	4,096
Total Activity(AU)	235,520	180,224	163,840	131,072	20,480
Specific Activity (AU/mg)	627	6,159	14,209	81,853	24,381
Activity Recovered (%)	100.0	76.5	69.6	55.7	8.7
Purification (fold)	1.0	9.8	22.6	130.5	38.9

TABLE 9

PURIFICATION OF BACTERIOCIN PRODUCED BY *LACTOBACILLUS ACIDOPHILUS* FR2

	Spent Broth	Precipitate	Gel Filtration	Micro-concentrate	Ion exchange
Volume	1,500.0	10.5	17.0	0.5	5.0
Protein Concn.(mg/ml)	0.20	0.94	0.57	2.66	0.14
Total Protein(mg)	306.0	9.9	9.7	1.3	0.71
Bacteriocin Activity (AU/ml)	8	1,024	512	16,384	256
Total Activity(AU)	12,000	10,752	8,704	8,192	1,280
Specific Activity (AU/mg)	39	1,089	797	6,154	1,802
Activity Recovered (%)	100.0	89.6	72.5	68.3	10.7
Purification (fold)	1.0	27.8	22.9	156.9	46.0

TABLE 10

PURIFICATION OF BACTERIOCIN PRODUCED BY *LACTOBACILLUS ACIDOPHILUS* A4

	Spent Broth	Precipitate	Gel Filtration	Micro-concentrate	Ion exchange
Volume	1,900.0	10.5	18.0	0.5	5.0
Protein Concn.(mg/ml)	0.20	1.26	0.71	4.03	0.19
Total Protein(mg)	387.6	13.2	12.7	2.0	0.96
Bacteriocin Activity (AU/ml)	512	65,536	32,768	1,048,576	16,384
Total Activity(AU)	972,800	688,128	589,824	524,288	81,920
Specific Activity (AU/mg)	2,509	52,012	46,400	260,076	85,333
Activity Recovered (%)	100.0	70.7	60.6	53.9	8 4
Purification (fold)	1.0	20.7	18.5	103.6	34.0

TABLE 11

PURIFICATION OF BACTERIOCIN PRODUCED BY *LACTOBACILLUS ACIDOPHILUS* GP1B

	Spent Broth	Precipitate	Gel Filtration	Micro-concentrate	Ion exchange
Volume	1,720.0	11.0	18.0	0.5	5.0
Protein Concn.(mg/ml)	0.19	1.26	0.76	3.12	0.19
Total Protein(mg)	326.8	13.9	13.6	1.6	0.93
Bacteriocin Activity (AU/ml)	64	8,192	4,098	131,072	2,048
Total Activity(AU)	110,080	90,112	73,764	65,536	10,140
Specific Activity (AU/mg)	336	6,501	5.404	42,010	11,010
Activity Recovered (%)	100.0	81.9	67.0	59.5	9.3
Purification (fold)	1.0	19.3	16.0	124.7	32.7

TABLE 12

PURIFICATION OF BACTERIOCIN PRODUCED BY *LACTOBACILLUS ACIDOPHILUS* GP2A

	Spent Broth	Precipitate	Gel Filtration	Micro-concentrate	Ion exchange
Volume	1,850.0	12.0	20.0	0.3	5.0
Protein Concn.(mg/ml)	0.12	0.86	0.50	2.69	0.13
Total Protein(mg)	222.93	10.26	10.00	0.81	0.67
Bacteriocin Activity (AU/ml)	512	65,536	32,768	2,079,152	32,768
Total Activity(AU)	947,200	786,432	655,360	629,146	163,840
Specific Activity (AU/mg)	4,249	76,650	655,360	779,610	24,5049
Activity Recovered (%)	100.0	83.0	69.2	66.4	17.3
Purification (fold)	1.0	18.0	15.4	183.5	57.7

TABLE 13

PURIFICATION OF BACTERIOCIN PRODUCED BY *LACTOBACILLUS ACIDOPHILUS*

	Spent Broth	Precipitate	Gel Filtration	Micro-concentrate	Ion exchange
Volume	920.0	12.0	20.0	0.5	5.0
Protein Concn.(mg/ml)	0.09	0.93	0.50	2.06	0.15
Total Protein(mg)	85.2	11.2	9.9	1.0	0.75
Bacteriocin Activity (AU/ml)	1,024	65,536	32,768	1,048,576	16,384
Total Activity(AU)	942,080	786,432	655,360	524,288	81,920
Specific Activity (AU/mg)	11,058	70,204	65,878	509,017	109,226
Activity Recovered (%)	100.0	83.5	69.6	55.7	8.7
Purification (fold)	1.0	6.3	6.0	46.0	9.9

TABLE 14

PURIFICATION OF BACTERIOCIN PRODUCED BY *LACTOBACILLUS ACIDOPHILUS* RP42

	Spent Broth	Precipitate	Gel Filtration	Micro-concentrate	Ion exchange
Volume	1,900.0	12.0	20.0	0.5	5.0
Protein Concn.(mg/ml)	0.19	1.24	0.63	3.43	0.17
Total Protein(mg)	361.0	14.9	12.7	1.7	0.87
Bacteriocin Activity (AU/ml)	8	1,024	512	16,384	256
Total Activity(AU)	15,200	12,288	10,240	8,192	1,280
Specific Activity (AU/mg)	42	825	808	4776	1,471
Activity Recovered (%)	100.0	80.8	67.4	53.9	8.4
Purification (fold)	1.0	19.6	19.2	113.4	34.9

APPENDIX B

ELUTION PROFILE OF AMMONIUM SULFATE

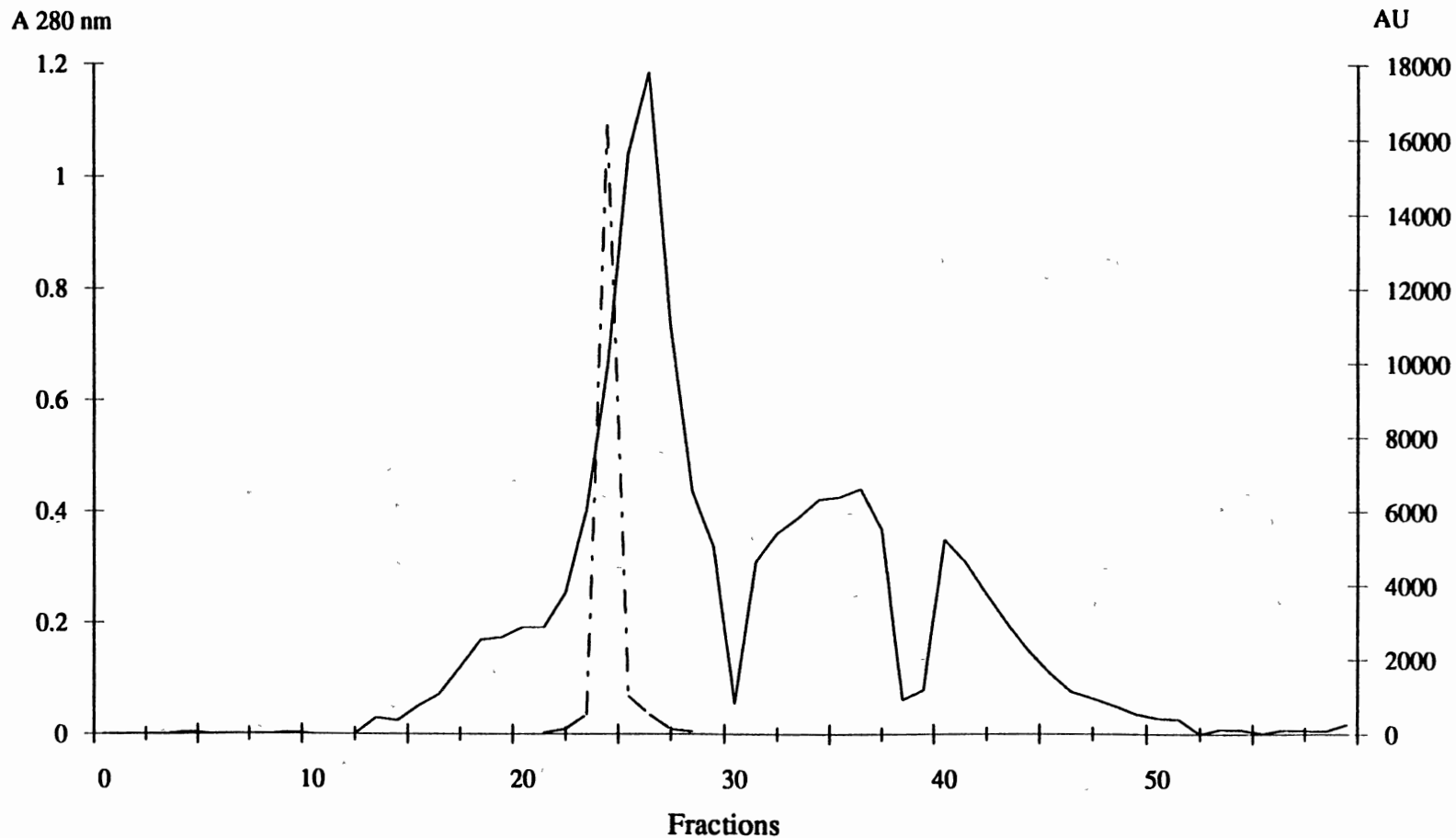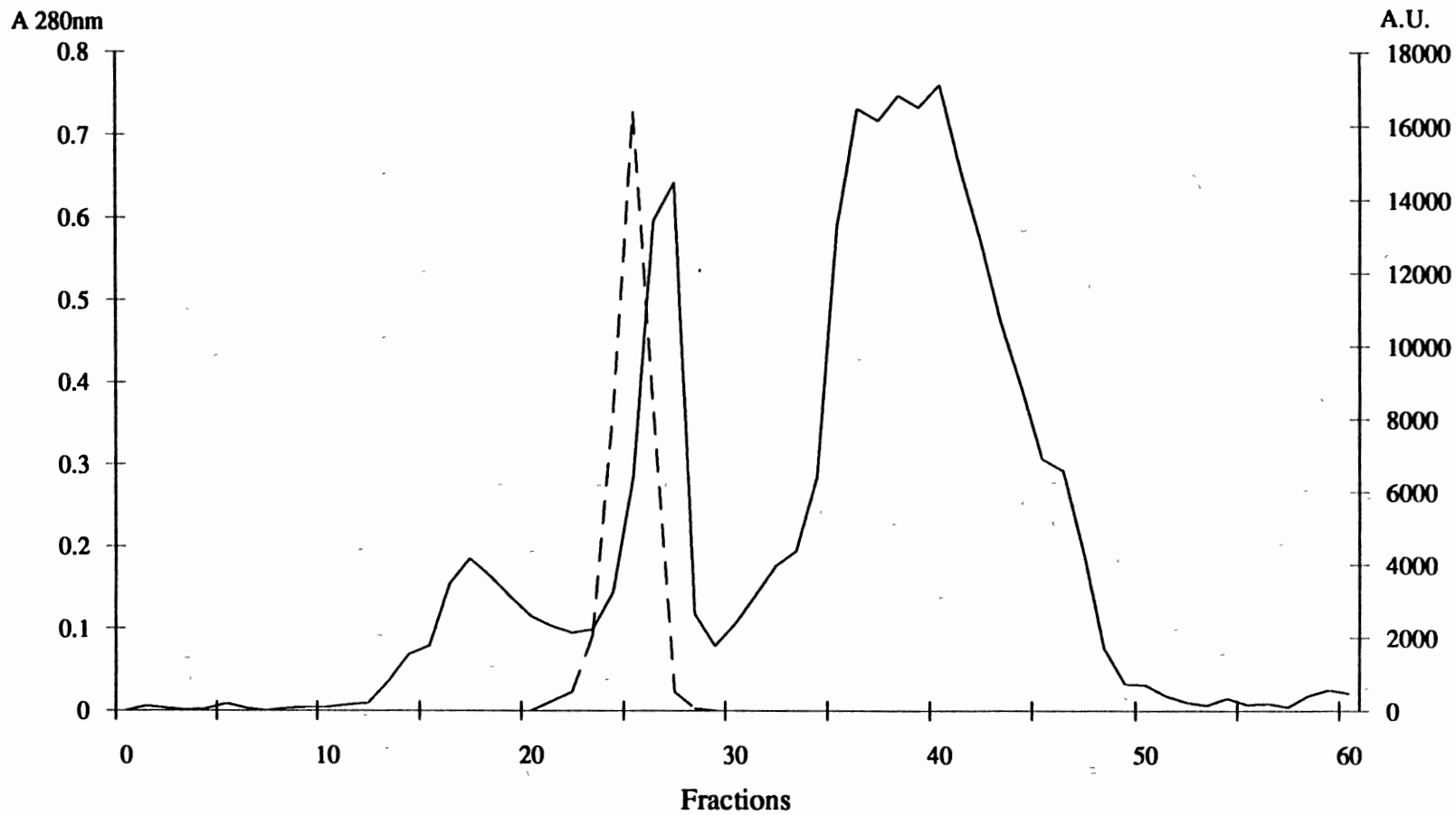PRECIPITATE OF INHIBITORY ACTIVITY ON

SEPHADEX G-200 GEL CHROMATOGRAPHY
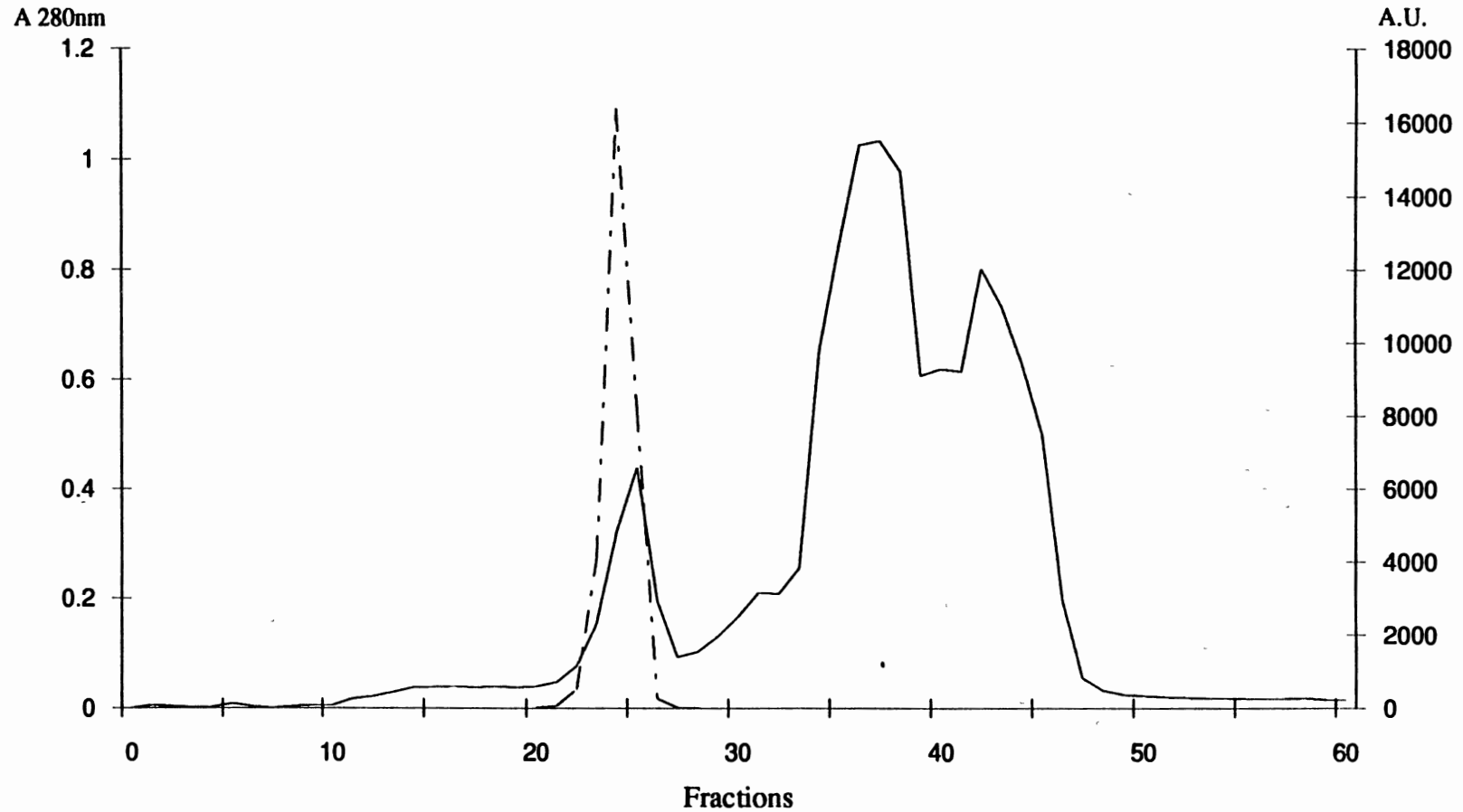
Figure 5. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* 1-3 on Sephadex G-200 in the presence of 0.1% SDS. Each 5 ml fraction was monitored at 280 nm (——) and was assayed for inhibitory activity (- - - -).
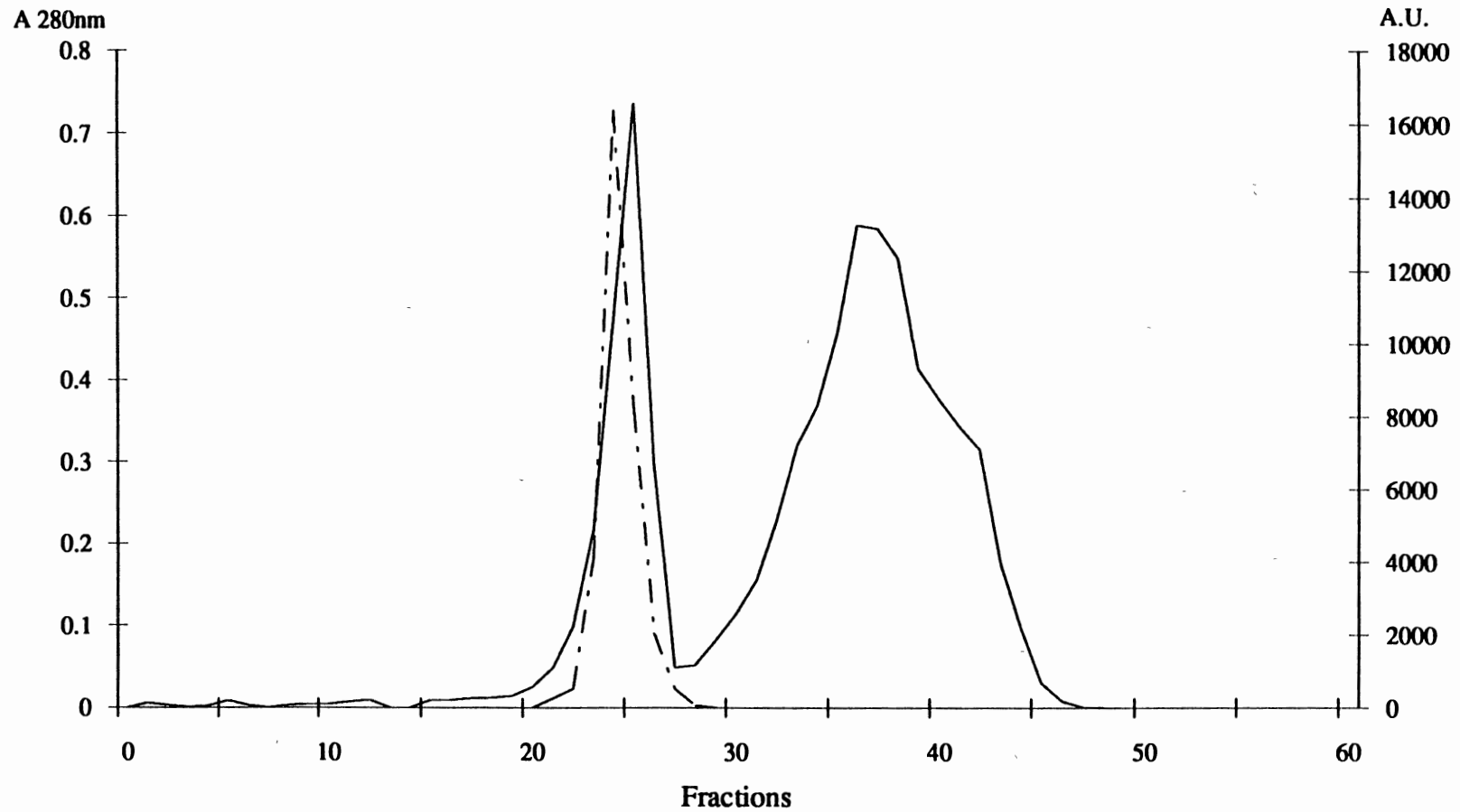
Figure 6. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* A1 on Sephadex G-200 in the presence of 0.1% SDS. Each 5 ml fraction was monitored at 280 nm (——) and was assayed for inhibitory activity (- - - -).
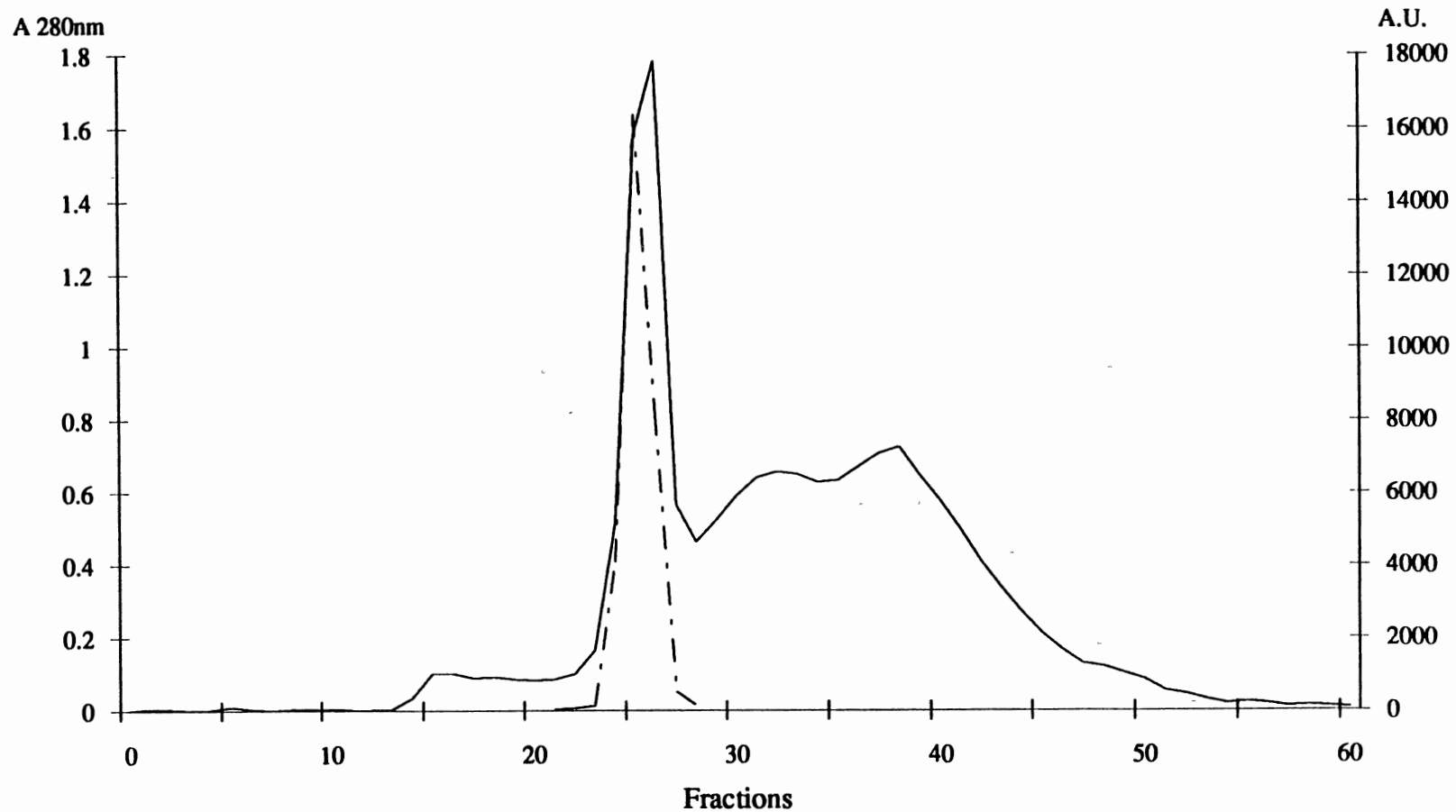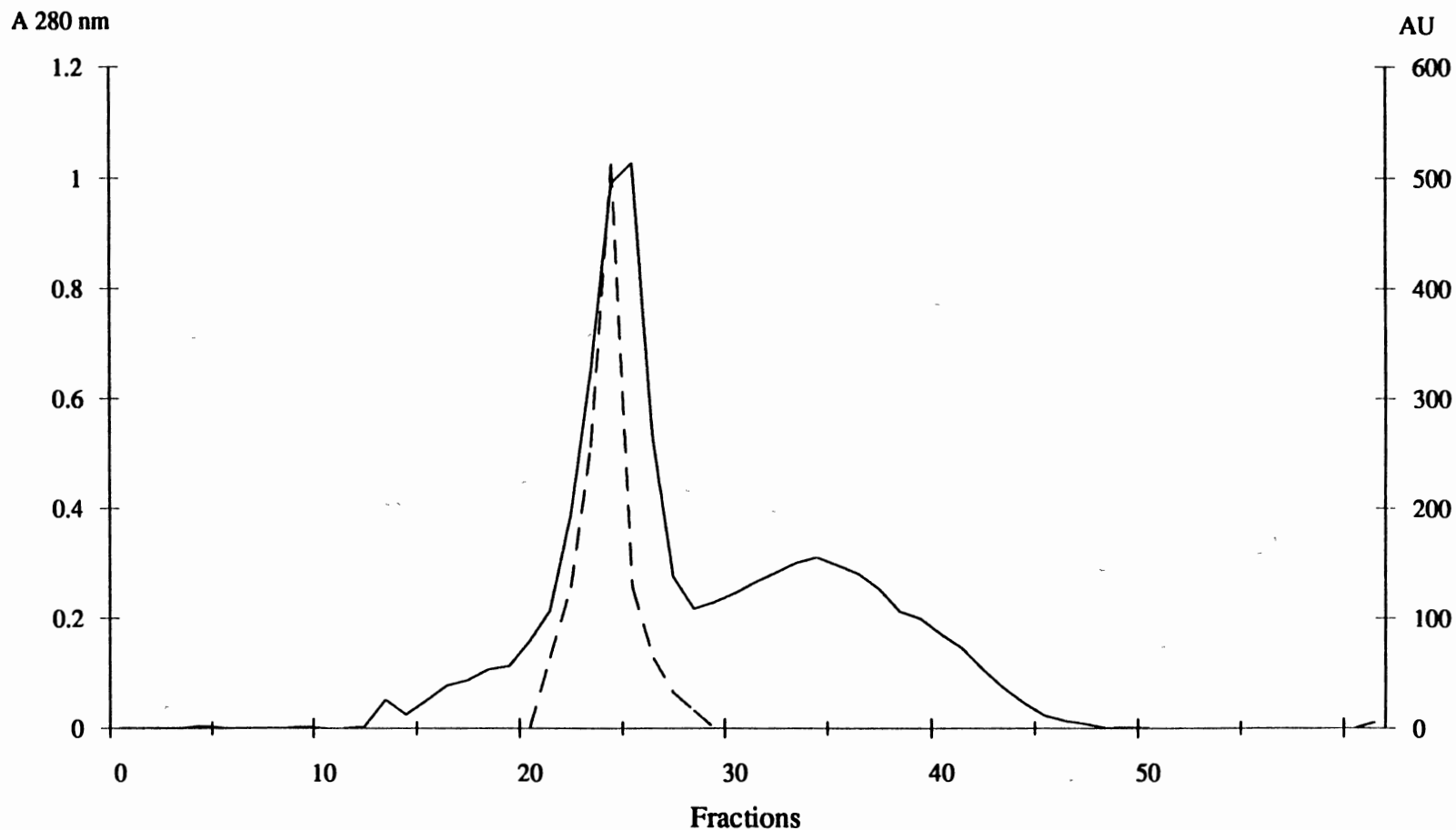
Figure 7. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* A4 on Sephadex G-200 in the presence of 0.1% SDS. Each 5 ml fraction was monitored at 280 nm (———) and was assayed for inhibitory activity (- - - -).
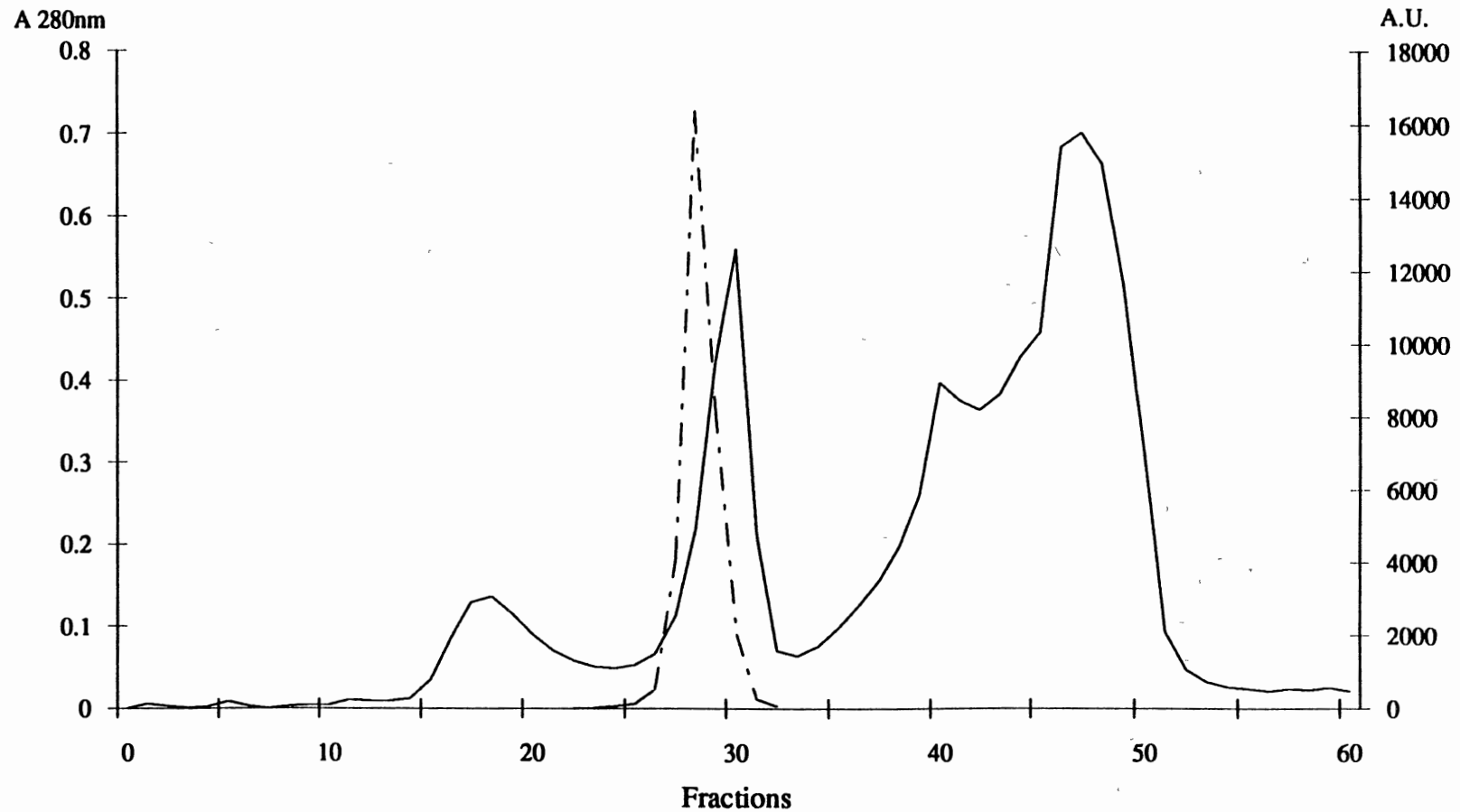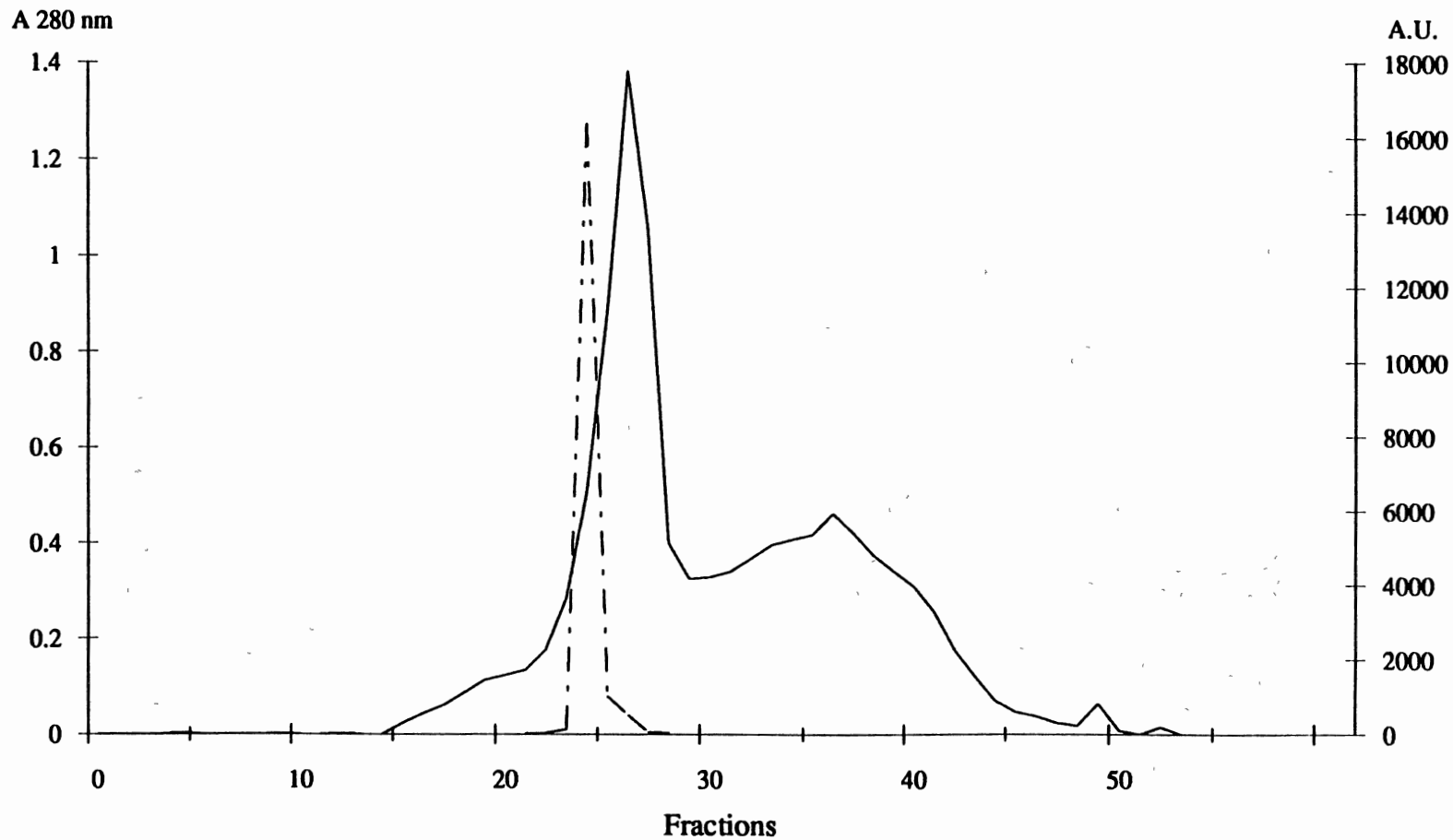
Figure 8. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* GP1A on Sephadex G-200 in the presence of 0.1% SDS. Each 5 ml fraction was monitored at 280 nm (——) and was assayed for inhibitory activity (- - - -).
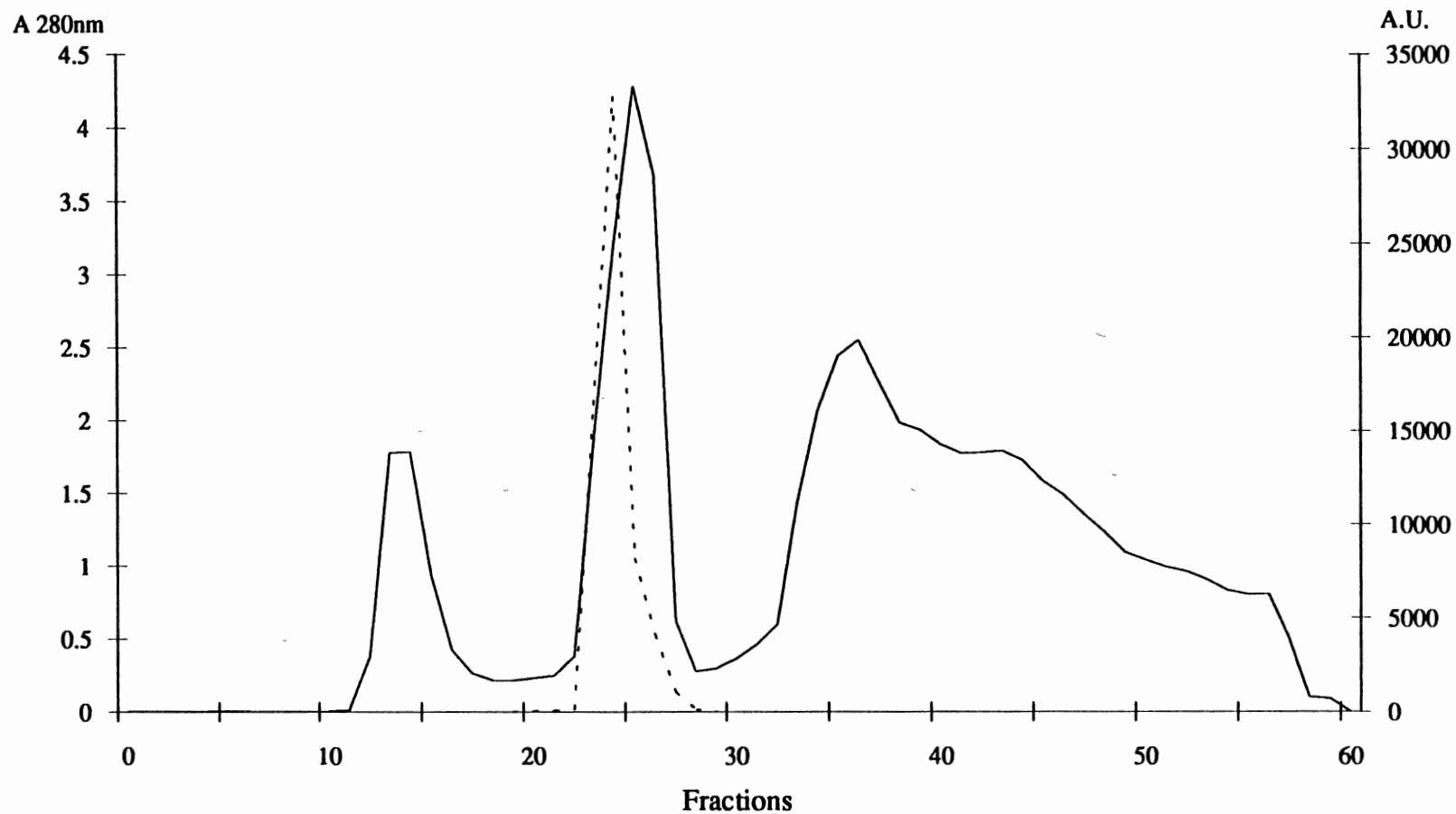
Figure 9. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* GP2A on Sephadex G-200 in the presence of 0.1% SDS. Each 5 ml fraction was monitored at 280 nm (———) and was assayed for inhibitory activity (- - - -).
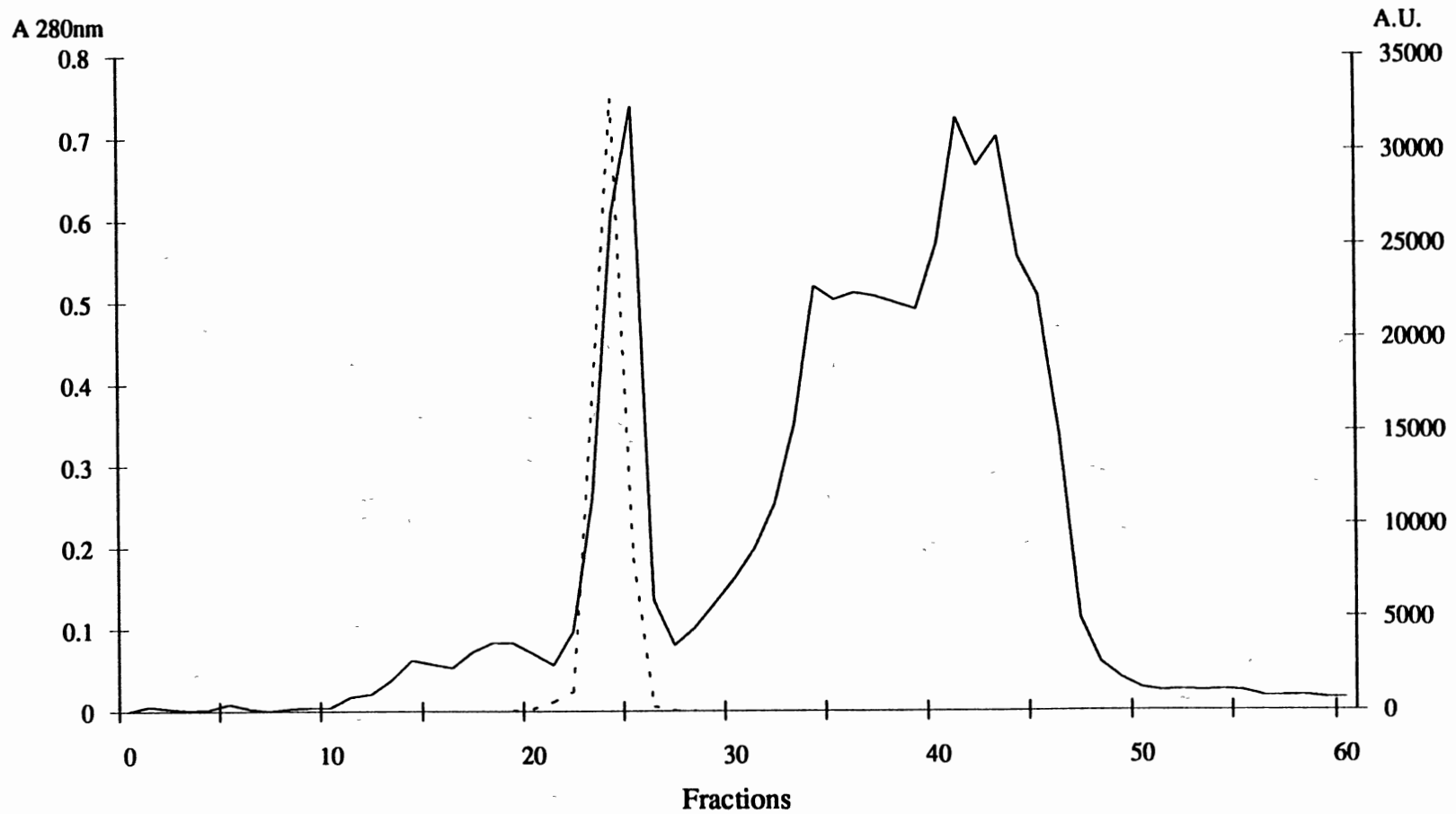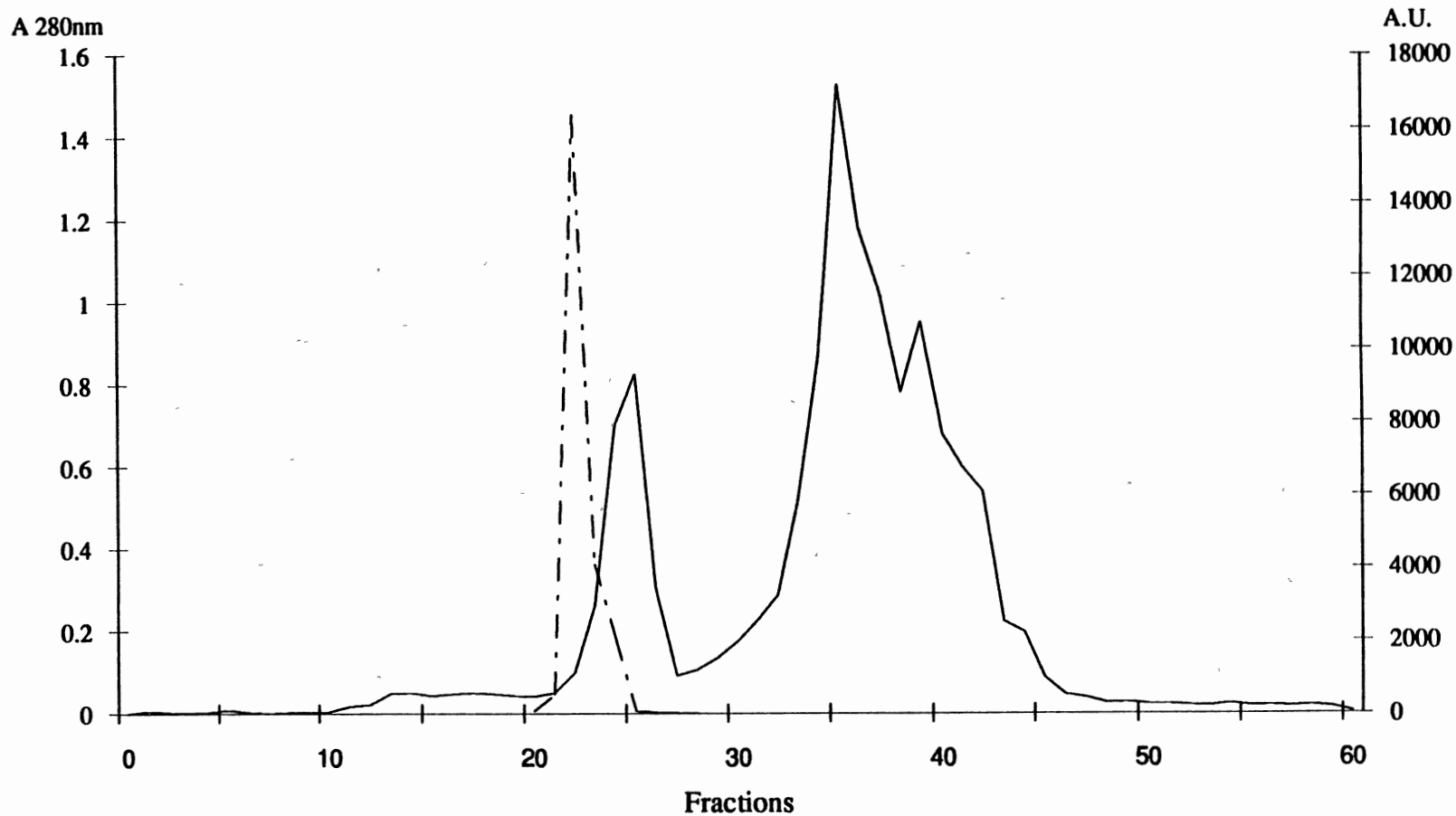
Figure 10. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* GP3A on Sephadex G-200 in the presence of 0.1% SDS. Each 5 ml fraction was monitored at 280 nm (————) and was assayed for inhibitory activity (- - - -).
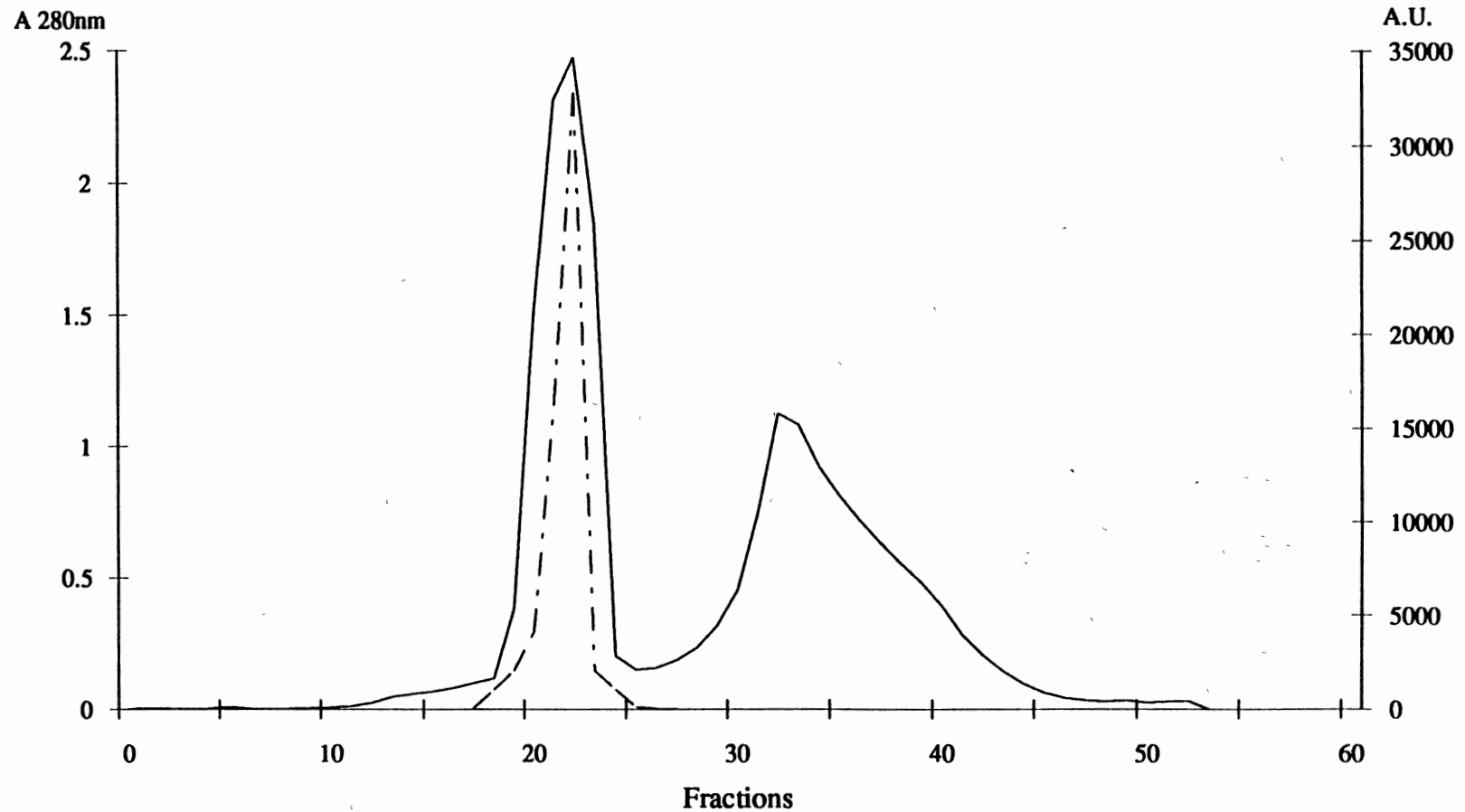
Figure 11. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* GP1B on Sephadex G-200 in the presence of 0.1% SDS. Each 5 ml fraction was monitored at 280 nm (———) and was assayed for inhibitory activity (- - - -).

Figure 12. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* RP34 on Sephadex G-200 in the presence of 0.1% SDS. Each 5 ml fraction was monitored at 280 nm (———) and was assayed for inhibitory activity (- - - -).
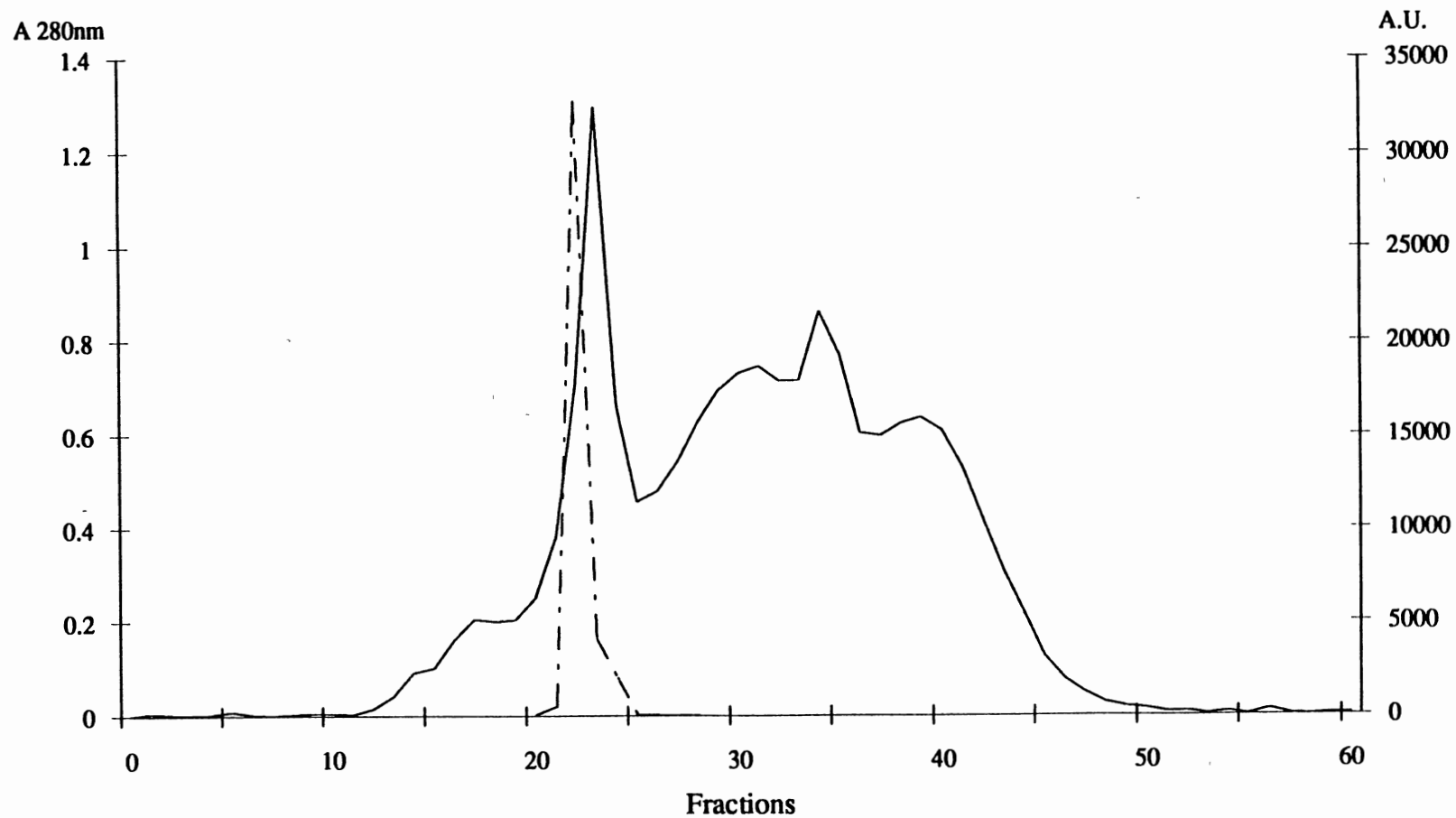
Figure 13. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* RP42 on Sephadex G-200 in the presence of 0.1% SDS. Each 5 ml fraction was monitored at 280 nm (——) and was assayed for inhibitory activity (- - - -).

Figure 14. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* 30SC on Sephadex G-200 in the presence of 0.1% SDS. Each 5 ml fraction was monitored at 280 nm (——) and was assayed for inhibitory activity (- - - -).

Figure 15. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* 381-IL-28 on Sephadex G-200 in the presence of 0.1% SDS. Each 5 ml fraction was monitored at 280 nm (——) and was assayed for inhibitory activity (- - - -).

Figure 16. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* FR2 on Sephadex G-200 in the presence of 0.1% SDS. Each 5 ml fraction was monitored at 280 nm (————) and was assayed for inhibitory activity (- - - -).

Figure 17. Elution profile of ammonium sulfate precipitate of inhibitory activity produced by *L. acidophilus* 606 on Sephadex G-200 in the presence of 0.1% SDS. Each 5 ml fraction was monitored at 280 nm (———) and was assayed for inhibitory activity (- - - -).

APPENDIX C

CHROMATOGRAPHY OF INHIBITORY ACTIVITY

OBTAINED FROM GEL CHROMATOGRAPHY ON
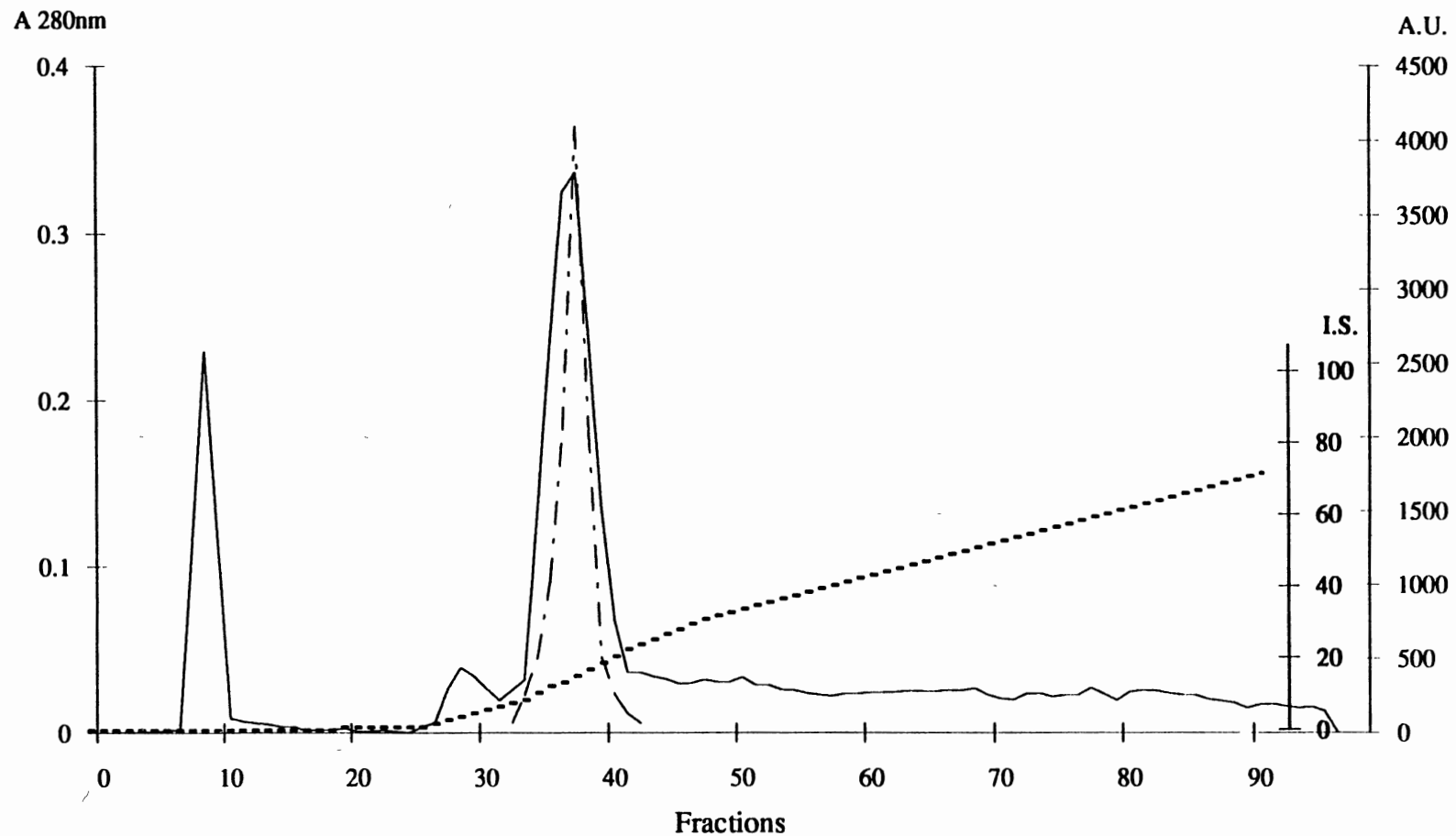
DE52 ANION EXCHANGE
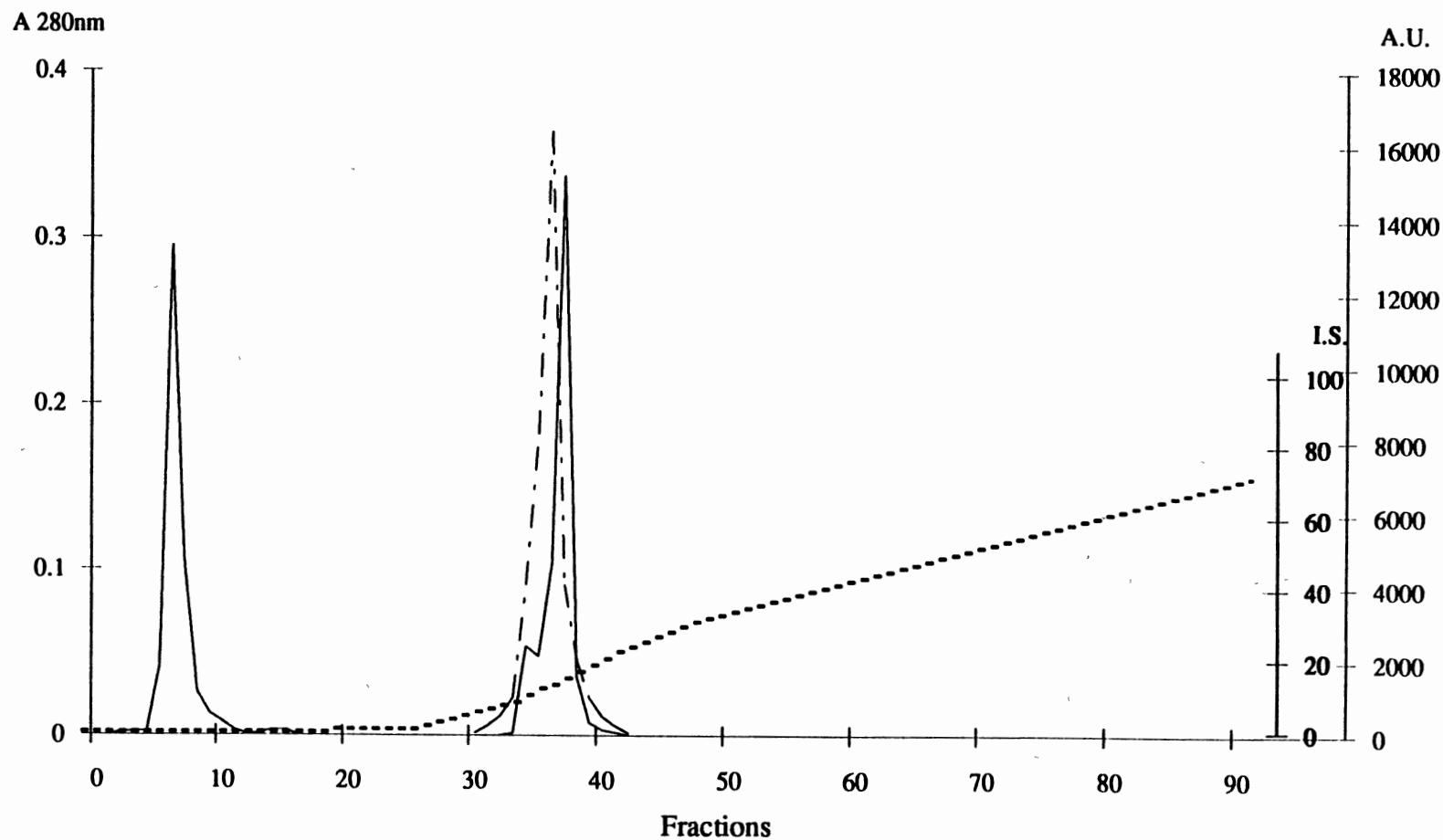
Figure 18. Chromatography of inhibitory activity obtained from Sephadex G-200 produced by *L. acidophilus* 606 on DE 52 anion exchange. The activity was eluted with 0 to 1.5 M NaCl. Each 4 ml fraction was monitored for absorbance at 280 nm (——) and for conductivity (-----), and was assayed for inhibitory activity (— – —).
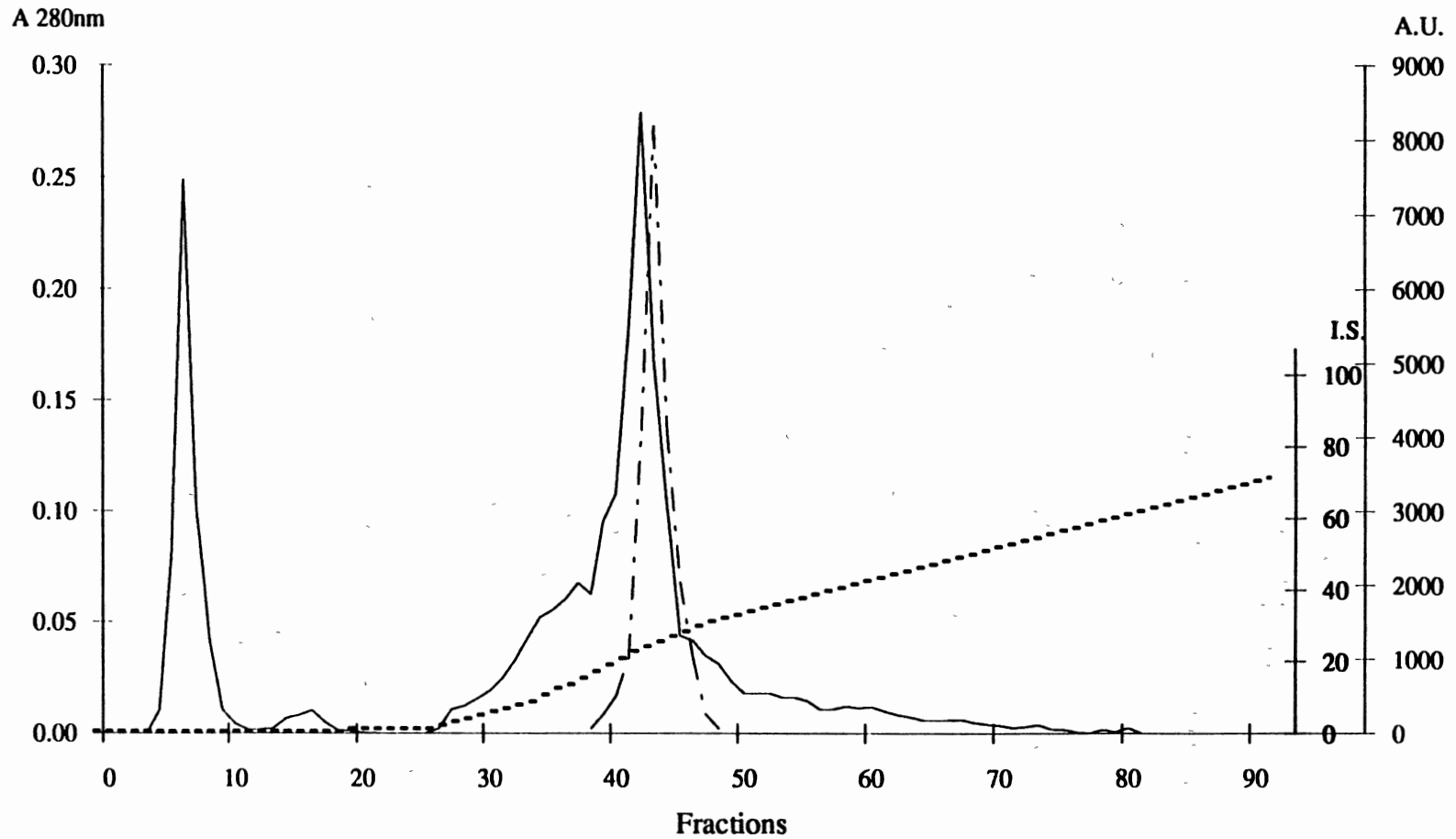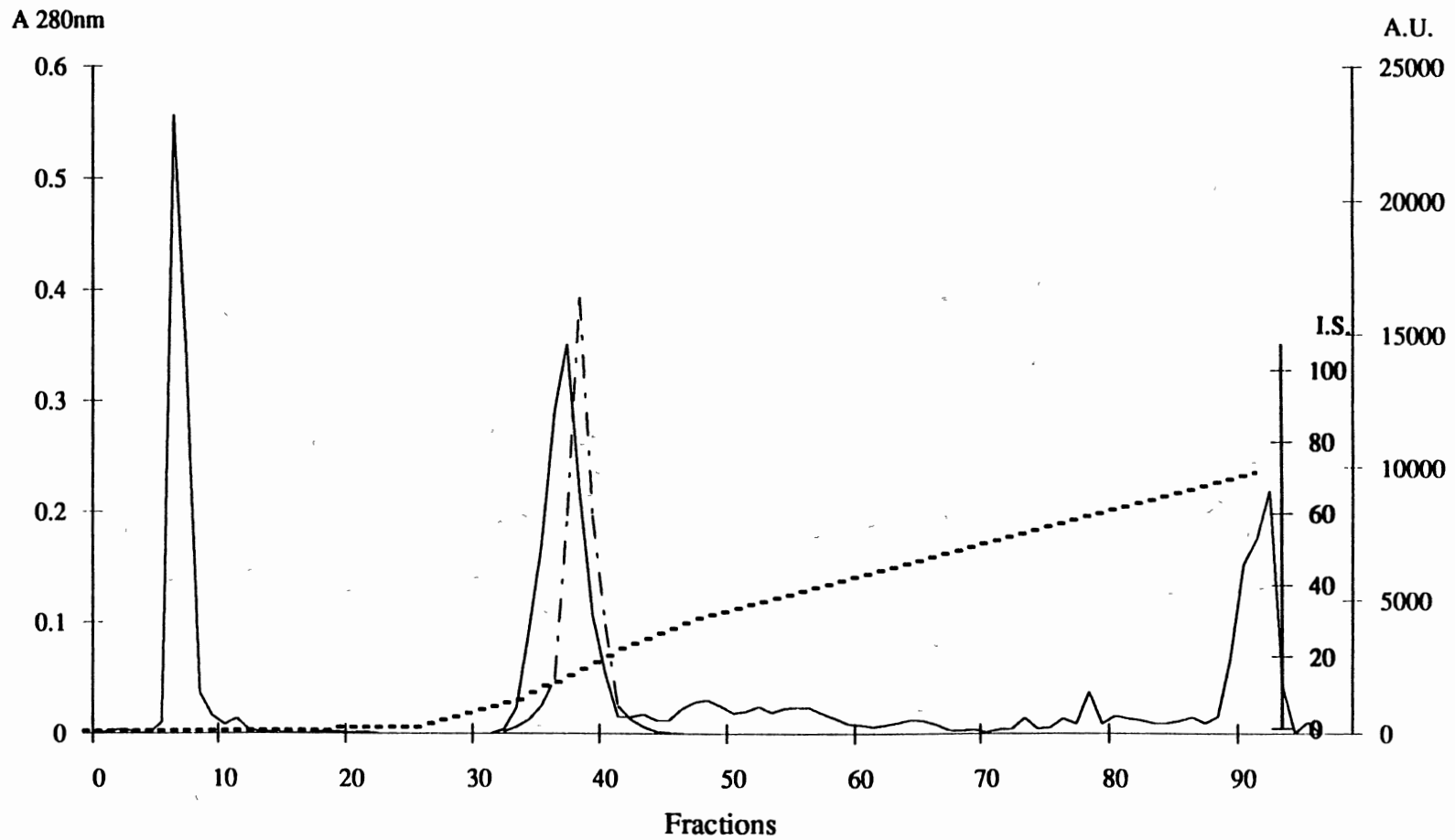
Figure 19. Chromatography of inhibitory activity obtained from Sephadex G-200 produced by *L. acidophilus* 30SC on DE 52 anion exchange. The activity was eluted with 0 to 1.5 M NaCl. Each 4 ml fraction was monitored for absorbance at 280 nm (⎯⎯) and for conductivity (-----), and was assayed for inhibitory activity (— – —).

Figure 20. Chromatography of inhibitory activity obtained from Sephadex G-200 produced by *L. acidophilus* FR2 on DE 52 anion exchange. The activity was eluted with 0 to 1.5 M NaCl. Each 4 ml fraction was monitored for absorbance at 280 nm (———) and for conductivity (-----), and was assayed for inhibitory activity (— – —).
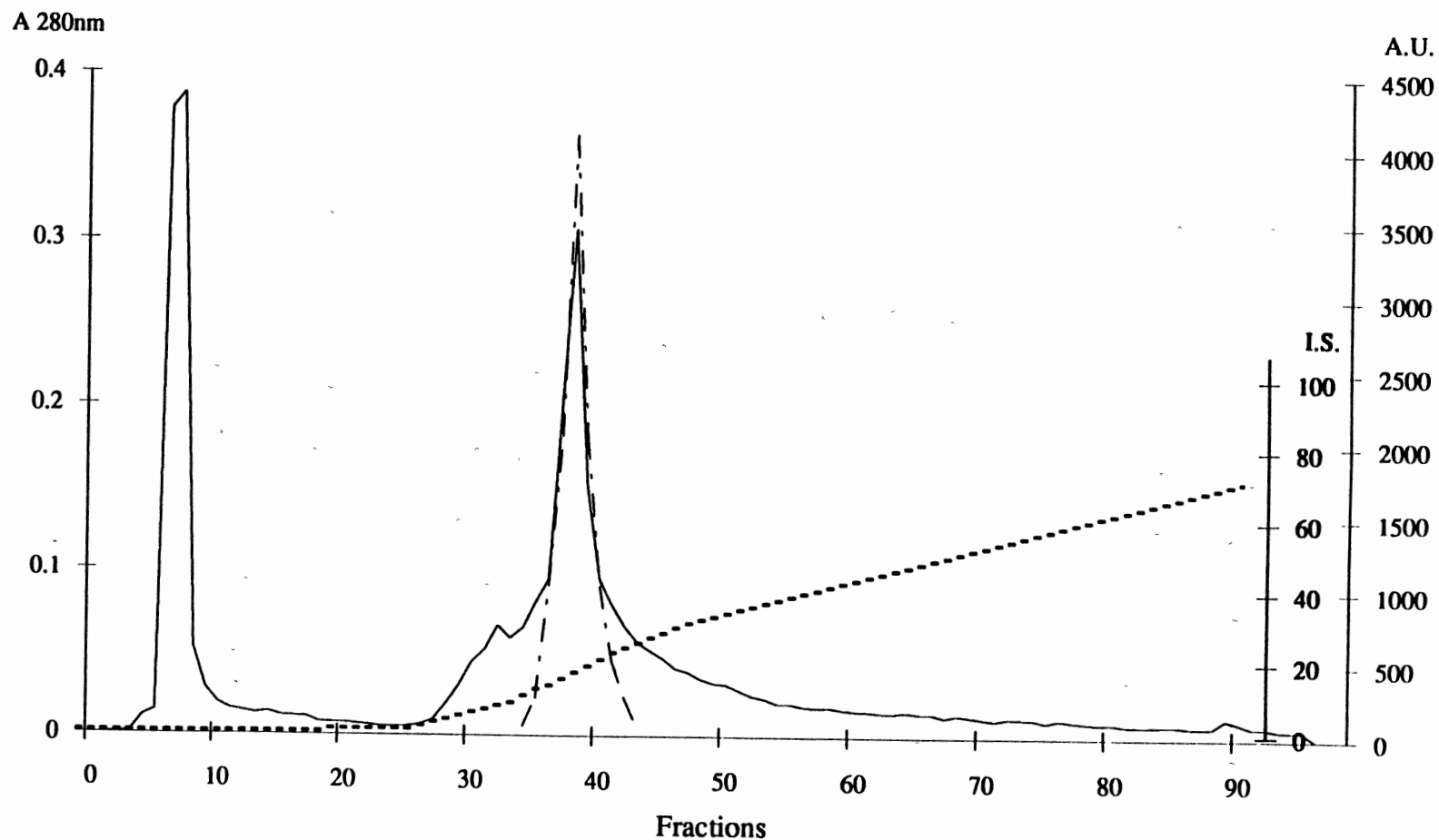
Figure 21. Chromatography of inhibitory activity obtained from Sephadex G-200 produced by *L. acidophilus* A4 on DE 52 anion exchange. The activity was eluted with 0 to 1.5 M NaCl. Each 4 ml fraction was monitored for absorbance at 280 nm (———) and for conductivity (-----), and was assayed for inhibitory activity (— – —).
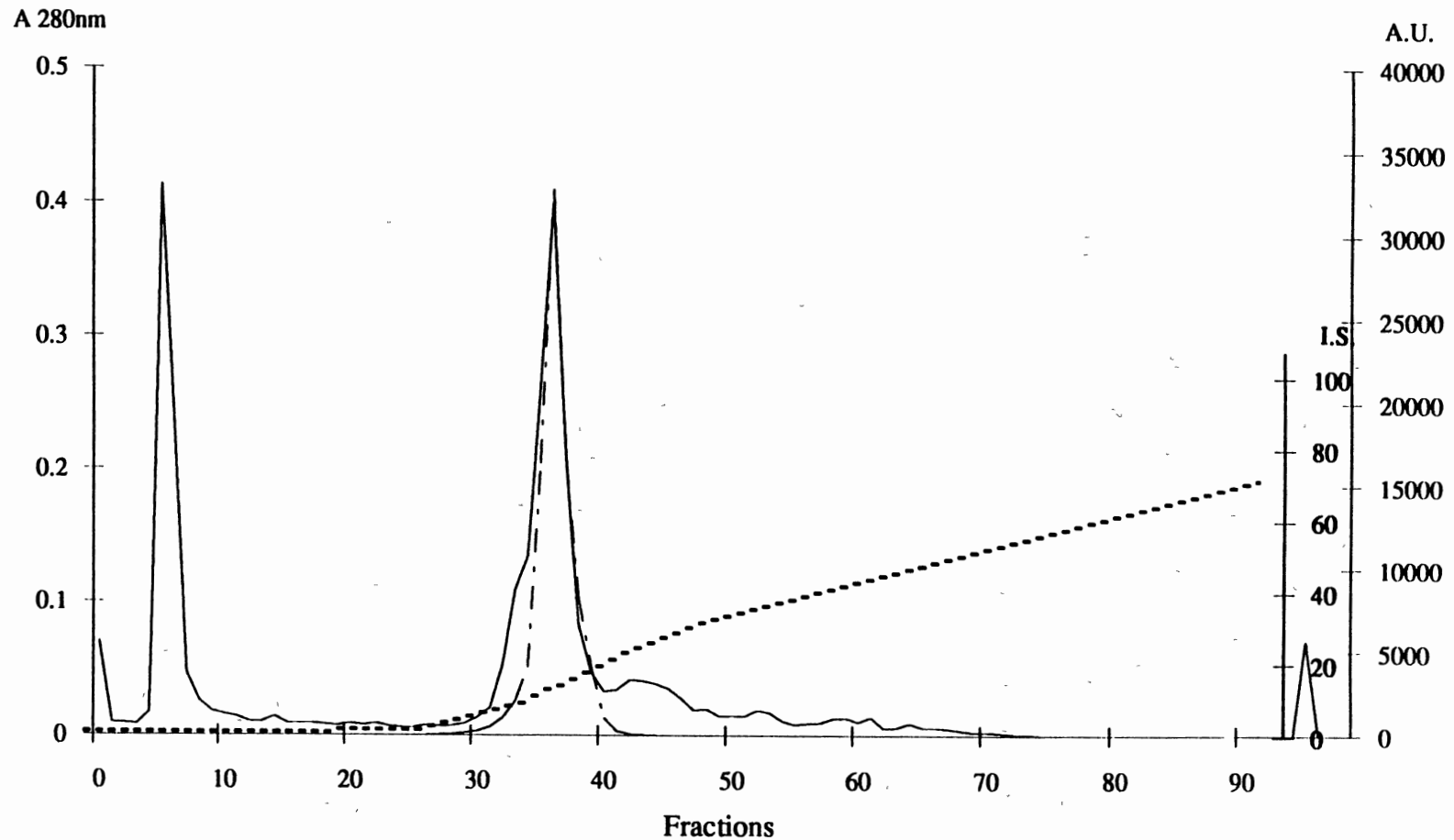
Figure 22. Chromatography of inhibitory activity obtained from Sephadex G-200 produced by *L. acidophilus* GP1B on DE 52 anion exchange. The activity was eluted with 0 to 1.5 M NaCl. Each 4 ml fraction was monitored for absorbance at 280 nm (———) and for conductivity (-----), and was assayed for inhibitory activity (— – —).

Figure 23. Chromatography of inhibitory activity obtained from Sephadex G-200 produced by *L. acidophilus* GP2A on DE 52 anion exchange. The activity was eluted with 0 to 1.5 M NaCl. Each 4 ml fraction was monitored for absorbance at 280 nm (———) and for conductivity (-----), and was assayed for inhibitory activity (— – —).
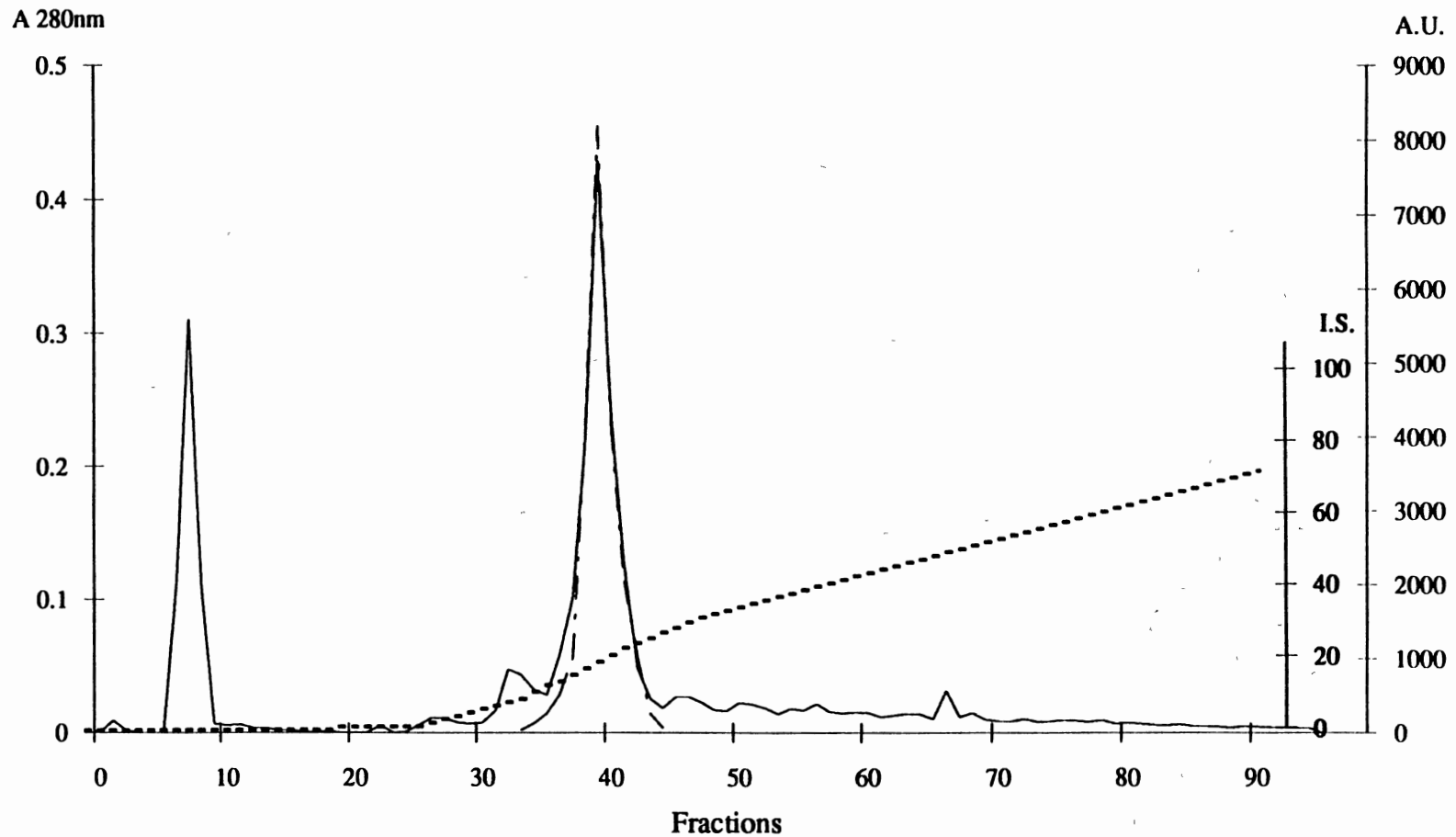
Figure 24. Chromatography of inhibitory activity obtained from Sephadex G-200 produced by *L. acidophilus* RP42 on DE 52 anion exchange. The activity was eluted with 0 to 1.5 M NaCl. Each 4 ml fraction was monitored for absorbance at 280 nm (————) and for conductivity (-----), and was assayed for inhibitory activity (— – —).

VITA

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