

Robert Cohen
560 Oradell Ave.
Oradell, NJ 07649
Tel: (201) 599-0325
Fax: (201) 599-0338

January 10, 2000

Dockets Management Branch
Food and Drug Administration
Department of Health and Human Services
Room 1-23
12420 Parklawn Dr.
Rockville, MD 20857

CITIZEN PETITION

The undersigned submits this petition under the Federal Food, Drug, and Cosmetic Act to request the Commissioner of Food and Drugs to challenge FDA's concept of substantial equivalence as it may apply to genetically engineered foods.

I respectfully request that FDA not approve Upjohn's application for approval of rbGH.

1. Upjohn has submitted an application for approval of rbGH.
2. In 1990, FDA determined that Upjohn's version of the genetically engineered bovine growth hormone was identical to the naturally occurring pituitary extract (Juskevich & Guyer, *SCIENCE*, Aug. 24, 1990). In fact, that is not the case.
3. FDA has developed a policy of "SUBSTANTIAL EQUIVALENCE", which eases the burden upon applicants for genetically modified organisms so that they need not perform substantial research. That policy should not apply to Upjohn's application.
4. New evidence suggests that "freak amino acids" are produced when the bovine growth hormone is re-combined with E-coli bacteria (Bernard Violand, *Protein Science*, July 3, 1994).

CONCLUSION

In 1990, FDA reviewers concluded that Upjohn's version of the bovine growth hormone was identical to the naturally occurring bovine pituitary extract. While this conclusion was correct based upon 1990's technology, it is no longer correct based upon new tools and assays.

00P-0177

CP1

Genetic engineering has provided surprises. In 1990, FDA gave the opinion that an amino acid difference in the middle of a protein chain could have dire consequences. That is in fact what happened.

After Monsanto learned that they had created freak amino acids, in transcribing rbGH they developed a technique by which they filtered out any and all proteins containing the freak amino acid. In doing so, they fixed the error but changed the formula. In fact, the actual hormone (*Posilac*) that is now on the market is a completely different formula than the one that FDA reviewed and approved.

Upjohn has the burden to conduct the same testing and research that was required of Monsanto.

The undersigned certifies, that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which the petition relies, and that it includes representative data and information known to the petitioner which are unfavorable to the petition.

Signature 

Name of Petitioner Robert Cohen

Mailing Address 560 Oradell Ave. Oradell NJ 07649

Telephone 201-599-0325

Robert Cohen
560 Oradell Ave.
Oradell, NJ 07649
Tel: 201-599-0325
Fax: 201-599-0338

January 10, 2000

ENVIRONMENTAL IMPACT STATEMENT

I, Robert Cohen, respectfully request that my Citizen's Petition (Docket No. ____ - ____) be placed on an accelerated review process.

Milk is a hormonal delivery system. Invitro and invivo studies of milk proteins cannot adequately demonstrate their effects unless they are delivered in aqueous solutions containing the same buffering mechanisms contained in milk.

One milk hormone, IGF-I has been identified as a key factor in the growth and proliferation of breast cancer, prostate cancer and lung cancer. It is now known that IGF-I levels in humans increases by a factor of 10% after milk consumption.

Based upon the research submitted, it is clear that the concept of substantial equivalence should not be applied to Upjohn's application. To not do so would jeopardize the health and safety of American dairy consumers.

Sincerely,



Robert Cohen

Robert Cohen
560 Oradell Ave.
Oradell, NJ 07649
Tel: (201) 599-0325
Fax: (201) 599-0338

December 30, 1999

Dockets Management Branch
Food and Drug Administration
Department of Health and Human Services
Room 1-23
12420 Parklawn Dr.
Rockville, MD 20857

CITIZEN PETITION

The undersigned submits this petition under the Federal Food, Drug, and Cosmetic Act to request the Commissioner of Food and Drugs to challenge FDA's concept of substantial equivalence as it may apply to genetically engineered foods.

Upjohn has submitted an application for approval of rbGH.

In 1990, FDA determined that Upjohn's version of the genetically engineered bovine growth hormone was identical to the naturally occurring pituitary extract (Juskevich & Guyer, *SCIENCE*, Aug. 24, 1990). In fact, that is not the case.

In 1992, FDA developed a policy of "SUBSTANTIAL EQUIVALENCE", which eases the burden upon applicants for genetically modified organisms so that they need not perform substantial research.

New evidence suggests that "freak amino acids" are produced when the bovine growth hormone is re-combined with E-coli bacteria.

While Upjohn's application is considered to be "TOP SECRET", this petition must be applied to the review process for that application which officially "does not exist", but in reality does exist. Such approval for substantial equivalence cannot be given.

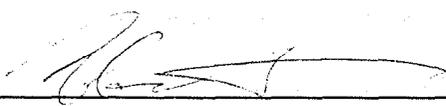
Attached to this petition is evidence that Monsanto's genetically engineered bovine growth hormone produced five freak amino acids and suggests that Upjohn's did the same.

Signature _____

Name of Petitioner _____

Mailing Address _____

Telephone _____



Robert Cohen

560 Oradell Ave. Oradell NJ 07649

201-599-0325

Isolation of *Escherichia coli* synthesized recombinant eukaryotic proteins that contain ϵ -N-acetyllysine

BERNARD N. VIOLAND,¹ MICHAEL R. SCHLITTLER,¹ CORY Q. LAWSON,¹
JAMES F. KANE,³ NED R. SIEGEL,² CHRISTINE E. SMITH,²
ERIC W. KOLODZIEJ,⁴ AND KEVIN L. DUFFIN²

¹Animal Sciences Division and ²Corporate Research Division, Monsanto Corporation, St. Louis, Missouri 63198
³SmithKline Beecham, King of Prussia, Pennsylvania 19406
⁴ARCO Chemical Co., Channelview, Texas 77530

(RECEIVED February 10, 1994; ACCEPTED April 20, 1994)

Lyndon - 33-507-1132

Abstract

Recombinant porcine (rpST) and bovine somatotropins (rbST) synthesized in *Escherichia coli* contain the amino acid, ϵ -N-acetyllysine. This amino acid was initially discovered in place of the normal lysine¹⁴⁴ in a modified reversed-phase HPLC (RP-HPLC) species of rpST. Mass spectrometry and amino acid sequencing of a tryptic peptide isolated from this RP-HPLC purified protein were used to identify this altered residue as ϵ -N-acetyllysine. Ion-exchange chromatography was utilized to prepare low isoelectric point (pI) forms of rpST and rbST, which are enriched in ϵ -N-acetyllysine. Electrospray mass spectrometry demonstrated that the majority of the protein in these low pI fractions contained species 42 Da larger than normal. Immobilized pH gradient electrophoresis (IPG) of the ion-exchange purified low pI proteins was used to isolate several monoacetylated species of rpST and rbST. The location of the acetylated lysine in each IPG-purified protein was determined by tryptic peptide mapping and amino acid sequencing of the altered tryptic peptides. Amino acid analyses of enzymatic digests of rpST and rbST were also used to confirm the presence of ϵ -N-acetyllysine in these recombinant proteins. These data demonstrate that a significant portion of rpST and rbST produced in *E. coli* contain this unusual amino acid.

Keywords: ϵ -N-acetyllysine; isoelectric focusing; mass spectrometry; peptide mapping; recombinant proteins; reversed-phase HPLC; somatotropins

The acetylation of the side-chain amino group of lysine residues in a polypeptide to form ϵ -N-acetyllysine was initially detected in calf thymus histones H3 and H4 (Allfrey et al., 1964, 1984; Gershoy et al., 1968; Delange et al., 1969). This modified amino acid has also been detected in other histones (Ruiz-Carrillo et al., 1976; Nelson, 1982). The acetyl donor group that is transferred to the ϵ -nitrogen of lysine by an acetyltransferase is acetyl-CoA (Allfrey et al., 1964; Nohara et al., 1966). This acetylation is a postsynthetic reaction that is not affected by inhibitors of protein synthesis (Allfrey et al., 1964, 1984).

The formation of ϵ -N-acetyllysine has been most thoroughly studied in histones and another class of DNA-binding proteins known as the high-mobility group (HMG) proteins (Sternier et al., 1979, 1981). Amino acid sequencing has shown that a specific set of lysines in the highly basic amino-terminal domains of these proteins is subjected to acetylation. The amount of ϵ -N-acetyllysine present in each protein is dynamic because deacetyl-

ating enzymes are also present in the cell nuclei. The function of acetylation-deacetylation is not completely understood, but this modification of histones and HMG proteins appears to be involved in the regulation of the interactions of these proteins with the negatively charged DNA molecules (Csordas, 1990).

Only a limited number of proteins besides histones and high-mobility proteins have been identified as containing ϵ -N-acetyllysine. These are a ferredoxin from *Halobacterium halobium* (Liss et al., 1978), α -tubulin from the unicellular green alga *Chlamydomonas reinhardtii* (L'Hernault & Rosenbaum, 1985), α -tubulin from 3T3 and HeLa cells (Piperno et al., 1987), and mouse neuronal α -tubulin (Edde et al., 1991). The functional role of ϵ -N-acetyllysine in ferredoxin is not understood, but this modification in α -tubulins converts this protein from the cell body form to the axonemal form. Monoclonal antibodies specific for ϵ -N-acetyllysine in α -tubulins have been used to study the appearance and disappearance of this amino acid in 3T3 and HeLa cells in culture (Piperno et al., 1987). The identification of this amino acid cannot be determined by simple amino acid analysis because the acetyl group is labile to the acidic or basic conditions normally used for hydrolysis. Instead,

Reprint requests to: Bernard N. Violand, Animal Sciences Division, Monsanto Corporation, 700 Chesterfield Village Parkway, St. Louis, Missouri 63198; e-mail: bnviol@cmail.monsanto.com.

complete enzymatic digestion to the component amino acids must be performed to identify ϵ -N-acetyllysine residues by amino acid analyses. Alternatively, ϵ -N-acetyllysine may be identified by amino acid sequencing using a phenylthiohydantoin (PTH)- ϵ -N-acetyllysine standard for comparison.

This amino acid has not been detected in either natural or recombinant proteins expressed in *Escherichia coli* except for 1 recent report (Harbour et al., 1992) describing its presence in a low pI fraction of recombinant bovine somatotropin (rbST). This work described the presence of ϵ -N-acetyllysine in several peptides isolated from a tryptic digest of this low pI rbST. Our work reports the identification of this amino acid in both rbST and recombinant porcine somatotropin (rpST), quantifies its level through amino acid analyses, and describes the isolation of several monoacetylated species of each of these proteins.

Results

rpST purified by salt-gradient elution on an ion-exchange resin can be resolved on reversed-phase HPLC (RP-HPLC) into at least 5 separate components (Fig. 1B). The first species has previously been identified as rpST containing a peptide bond chemically cleaved between Asn⁹⁹ and Ser¹⁰⁰, and the second one has been shown to contain rpST with isoaspartate⁹⁹ (Violand et al., 1990). The last eluting component (peak 5), which accounted for 5–10% of the total protein, was isolated by RP-HPLC (Fig. 1A) and analyzed by tryptic peptide mapping along with standard rpST (Fig. 2). All of the major peptides in the standard chromatogram have been previously sequenced and account for the entire sequence of this protein. These chromatograms show that the major difference between the modified protein and the standard rpST was the presence of a new peptide that eluted at 34.7 min (Fig. 2A, peak 2). The intensity of the normal peptide in this modified protein (peak 1, 29.7 min) was decreased

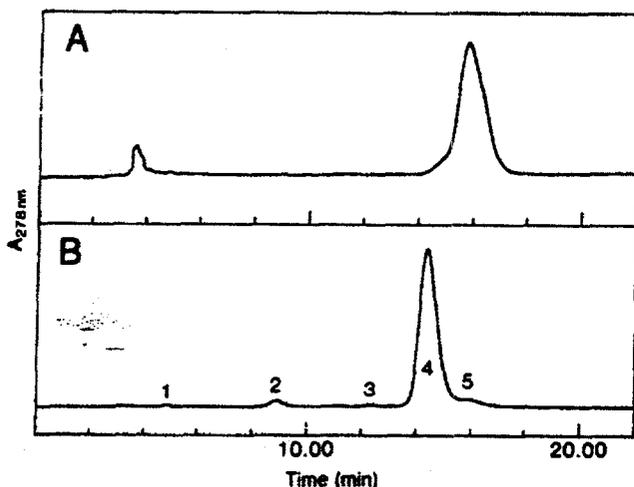


Fig. 1. Reversed-phase (RP)-HPLC analysis of (A) a purified late-eluting component and (B) recombinant porcine somatotropin (rpST). rpST purified from *E. coli* inclusion bodies by refolding and subsequent Phoenix ion-exchange chromatography was analyzed on a Vydac C-18 resin. Peak 1 contains rpST cleaved between Asn⁹⁹ and Ser¹⁰⁰, peak 2 contains rpST with isoaspartate⁹⁹, and the major component in peak 5 is rpST containing ϵ -N-acetyllysine¹⁴⁴. Peak 5 isolated by this HPLC method is shown in the upper chromatogram.

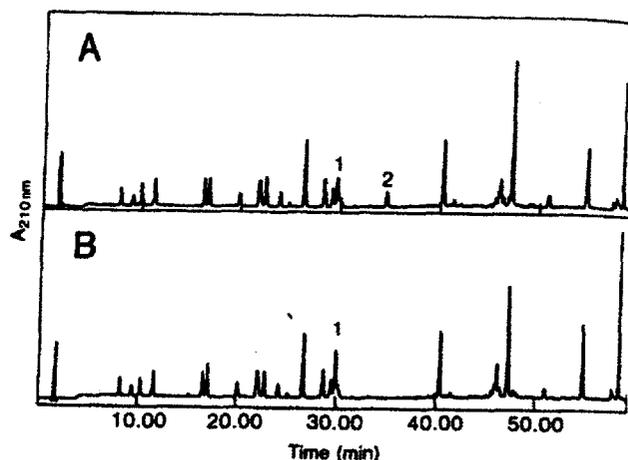


Fig. 2. RP-HPLC separation of the tryptic peptides of (A) peak 5 from Figure 1 and (B) normal rpST. Normal rpST and component 5 of rpST from Figure 1 were digested with trypsin and analyzed by RP-HPLC on a Nucleosil C-18 column. The peptide labeled 1 in both chromatograms contains the normal rpST residues 140–150, whereas the peptide labeled with a 2 in the upper chromatogram contains residues 140–150 with ϵ -N-acetyllysine substituted for lysine¹⁴⁴.

compared to the normal protein. The unidentified peptide and the normal peptide were isolated and subjected to amino acid sequencing. The results of these analyses demonstrated that peptide number 1 contains the normal rpST residues 140–150, which are Gln-Thr-Tyr-Asp-Lys-Phe-Asp-Thr-Asn-Leu-Arg. The Lys at residue 144 is not readily cleaved by trypsin probably because of the neighboring Asp residues. The fact that this Lys is not easily susceptible to trypsin cleavage has also been observed previously (Violand et al., 1990). Sequencing of the modified peptide yielded an identical sequence to the normal peptide of residues 140–150, except residue 144 was not a lysine and was also not identifiable as one of the normal PTH amino acid standards. Amino acid analyses of the normal and modified peptides yielded identical values for the 2 peptides. This information suggested that residue 144 in the modified peptide was probably a lysine that was altered by addition of a chemical group unstable to the acid conditions used for the amino acid hydrolysis.

Mass spectrometry analyses yielded a positive molecular ion of 1,442.8 for the modified peptide compared to 1,400.8 for the normal peptide of residues 140–150. This difference of 42 Da and the sequencing and amino acid analyses data suggested that the modified peptide may contain a lysine that was acetylated because 42 Da is the increase in mass of ϵ -N-acetyllysine compared to lysine. This modified amino acid was confirmed to be ϵ -N-acetyllysine because the retention time of its PTH derivative was identical to that of authentic PTH- ϵ -N-acetyllysine (Fig. 3). The above combination of techniques was used to demonstrate that the modified late-eluting rpST protein contained ϵ -N-acetyllysine¹⁴⁴. It can be observed, however, that there is a considerable amount of normal peptide of residues 140–150 present in the peptide map of the HPLC-purified late-eluting protein. From the peptide maps it was calculated that approximately 40% of the altered HPLC-purified protein contains ϵ -N-acetyllysine¹⁴⁴.

Because acetylation of a lysine should lower the pI of a protein, the HPLC-isolated modified protein was analyzed by iso-

Fig. 3. A peptide and PTH added, for the tryptophan acetyl

Electri-
meau-
of rpS
present
rpST v
only b
HPLC
rpST ;
the
had u
equal
on DE
major
in the
for IE
forms
in c
NEF b
de (IP
these l
an IP
4, Fig
bands
was st
of str
oral d
in Fig
purifi
100-1
in Fig
resid
place
cont
tides

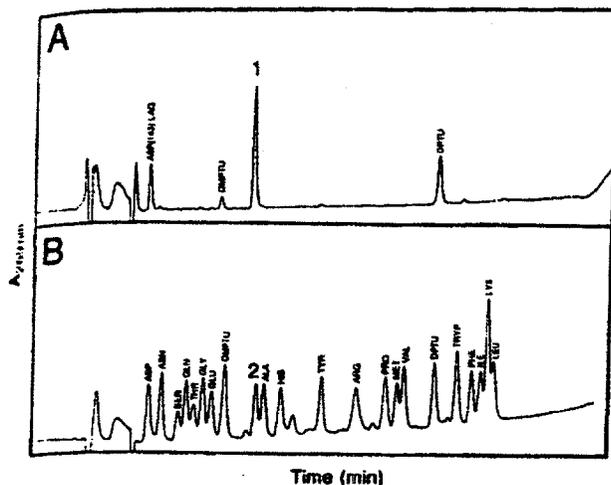


Fig. 3. RP-HPLC chromatograms of phenylthiohydantoin (PTH) standards and the PTH-amino acid from residue 144 in the modified rpST peptide from Figure 2. The lower HPLC chromatogram shows the normal PTH-amino acid standards to which PTH- ϵ -N-acetyllysine has been added, and the upper chromatogram shows the PTH-derivative obtained for the fifth residue (position 144) in the modified peptide obtained from the tryptic map in Figure 2A (peptide 2). This analysis shows that this unknown PTH-amino acid elutes at the same retention time as PTH- ϵ -N-acetyllysine.

electric focusing (IEF) and does have a lower pI as expected. Because ϵ -N-acetyllysine was present in this low pI component of rpST, the identity of the other low pI components that are present in this protein was investigated. The low pI species of rpST were separated from the majority of the standard pI protein by ion-exchange chromatography on DE-52 resin using a pH gradient (Wood et al., 1989). IEF analyses (Fig. 4) show the rpST applied to the DE-52 resin (Phoenix resin purified, lane 2), the normal pI rpST (lane 3), and low pI rpST (lane 4) purified using DE-52 resin. This IEF gel shows that there are several low pI species of rpST, and ion-exchange chromatography on DE-52 was effective in separating these components from the majority of the normal pI rpST. These low pI forms have pIs in the range of 6.1–6.4 compared to 7.4 for normal rpST. Similar IEF analysis of rbST has also shown the presence of low pI forms in this protein (Bogosian et al., 1989).

In order to determine the chemical identity of these different IEF bands, preparative immobilized pH gradient electrophoresis (IPG) was used to isolate sufficient quantities of several of these low pI bands for peptide mapping analyses. Figure 5 shows an IPG gel of the purified low pI rpST (same sample as in lane 4, Fig. 4) isolated by DE-52 chromatography and several of the bands purified using this technique. Each of these isolated bands was subjected to tryptic peptide mapping. Peptide maps (Fig. 6) of standard rpST and the rpST from lane 4 in Figure 5 show several differences. Two of the normal peptides (labeled 1 and 2 in Fig. 6B) are reduced in intensity in the map of this IPG-purified rpST. These peptides correspond to amino acid residues 168–171 and 172–177, respectively. A novel peptide is present in Figure 6A (peak 3). This peptide was sequenced and contains residues 168–177 with residue 171 containing ϵ -N-acetyllysine in place of lysine. These data show that this low pI form of rpST contains ϵ -N-acetyllysine¹⁷¹ and explains why the normal peptides of residues 168–171 and 172–177 are absent in this peptide

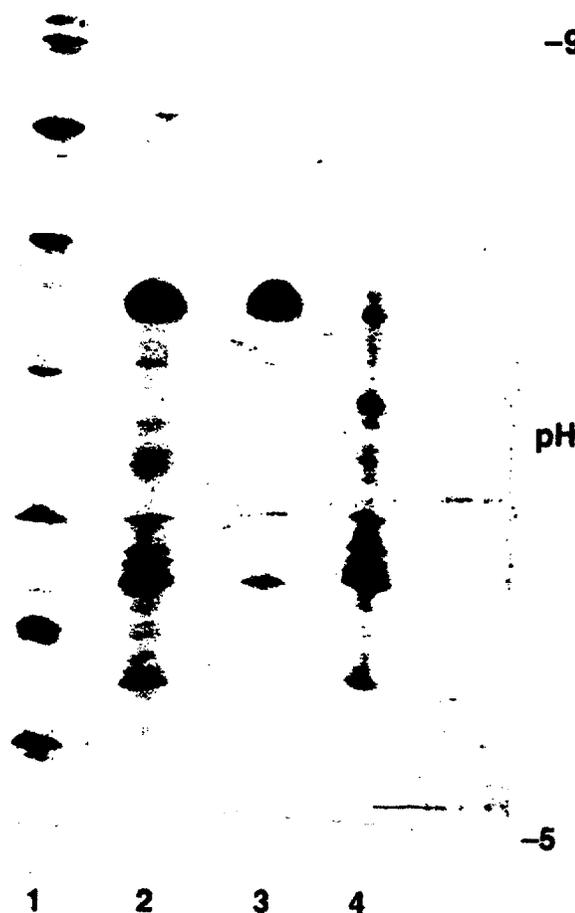


Fig. 4. Analytical isoelectric focusing of normal and low pI rpST purified using DE-52 chromatography. The rpST shown in lane 2 was separated by DE-52 chromatography into the normal (50 μ g, lane 3) and low pI protein (50 μ g, lane 4). Lane 1 contains Pharmacia pI standards.

map because trypsin cannot cleave the peptide bond between ϵ -N-acetyllysine¹⁷¹ and Ala¹⁷². Similar peptide mapping was performed on the 2 other isolated IPG rpST bands shown in Figure 5. These results demonstrated that the rpST in lane 3 contains ϵ -N-acetyllysine¹¹², and the band in lane 2 is rpST, which has the peptide bond between Asn⁹⁹ and Ser¹⁰⁰ cleaved. This cleaved product has been previously isolated by RP-HPLC and chemically characterized (Violand et al., 1990). Characterization of the RP-HPLC- and IPG-separated species shows that there are at least 3 acetylated species of rpST (ϵ -N-acetyllysine 144, 112, and 171), which are formed in *E. coli*. Similar analyses of rbST have shown that ϵ -N-acetyllysine exists at residues 144, 157, and 167. Harbour et al. (1992) have identified ϵ -N-acetylation as being present at residues 157, 167, 171, and 180 in rbST from the tryptic digest of their low pI rbST.

IPG was useful for separating several monoacetylated forms of rpST, but this method is not readily amenable for quantitation of ϵ -N-acetyllysine in rpST. The total amount of ϵ -N-acetyllysine was determined using total enzymatic digestion of the protein followed by amino acid analysis. For the low pI rpST (Fig. 4, lane 4), 0.42 mol ϵ -N-acetyllysine per mol of protein was obtained, whereas the normal pI rpST (Fig. 4, lane 3) prepared

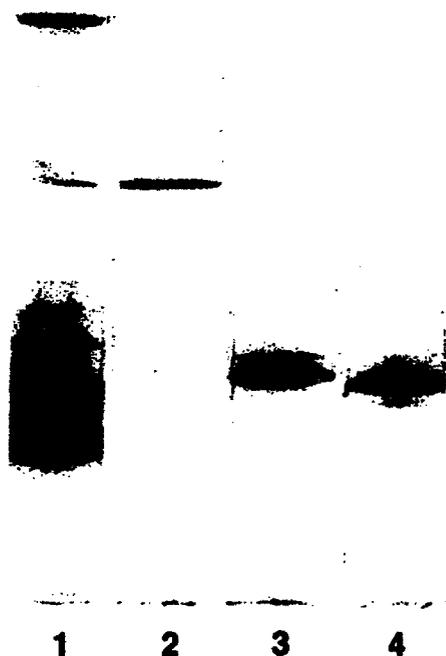


Fig. 5. Immobilized pH gradient electrophoresis (IPG) analyses of rpST (20 μ g each lane). IPG of the low pI rpST shown in lane 1 was used to isolate several different low pI bands in a homogeneous form as judged by this technique. Lane 4 contains rpST, which contains an ϵ -N-acetyllysine¹⁷¹; lane 3 contains rpST, which contains an ϵ -N-acetyllysine¹¹²; lane 2 contains rpST, which has the peptide bond between Asn⁹⁹ and Ser¹⁰⁰ cleaved.

by DE-52 chromatography yielded nondetectable levels (<0.05 mol/mol) of this amino acid. As expected, these data show that ϵ -N-acetyllysine is present only in the low pI components of rpST and not in the normal pI rpST. Assuming that each molecule contains only 1 ϵ -N-acetyllysine, then about 40% of the low pI proteins are acetylated. The remainder of the low pI species are mostly deamidated forms of rpST, such as the aspartate⁹⁹ and isoaspartate⁹⁹ species, which have been previously described (Violand et al., 1990).

It was also desirable to measure the amount of ϵ -N-acetyllysine in rpST and rbST isolated directly from inclusion bodies without going through the refold, Phoenix, and DE-52 ion-exchange steps. This was accomplished by reducing and carboxymethylating the free sulfhydryl groups in the proteins from inclusion bodies and subsequently purifying the desired somatotropin away from the *E. coli* proteins by RP-HPLC. The enzyme digestion procedure was then performed on this protein to determine the amount of ϵ -N-acetyllysine. These results yielded 0.22 and 0.27 mol of ϵ -N-acetyllysine per mol of rpST and rbST, respectively. A value of 0.23 mol of ϵ -N-acetyllysine per mol of protein was obtained for rpST (Fig. 4, lane 2) purified from the refolded protein isolated by the Phoenix ion-exchange chromatography. This value would be expected to be

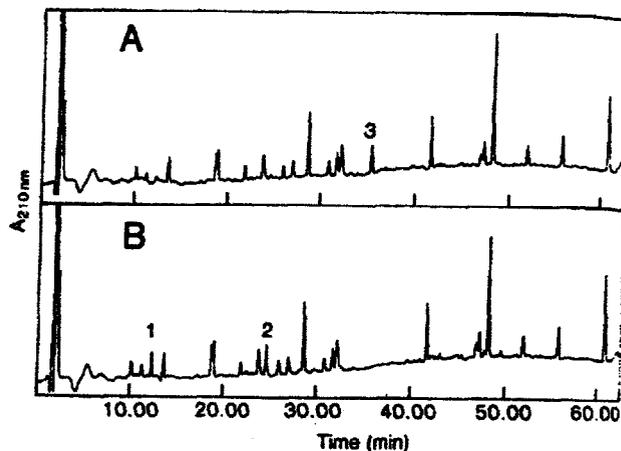


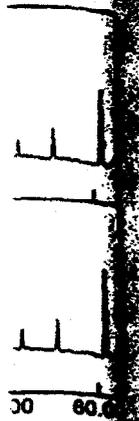
Fig. 6. RP-HPLC separation of the tryptic peptides for (A) rpST containing ϵ -N-acetyllysine¹⁷¹ and (B) normal rpST. The peptides labeled in the normal rpST chromatogram are peptide 1 containing residues 168-171 and peptide 2 containing residues 172-177. The chromatogram of rpST isolated by IPG shows an abnormal peptide (number 3), which contains residues 168-177 with ϵ -N-acetyllysine substituted for lysine¹⁷¹. Notice the reduction of the normal peptides 1 and 2 in the upper chromatogram.

lower than those obtained for the low pI protein, which should be enriched in this amino acid.

Electrospray mass spectrometry (ESMS) was also used to substantiate the presence of ϵ -N-acetyllysine in rbST and rpST. ESMS analyses (Fig. 7) of normal pI and low pI rpST yielded the expected mass of 21,798 Da for normal rbST, whereas the low pI rpST contains a majority of rpST, which is 42 Da higher in mass. Similar analyses (Fig. 8) of normal pI (8.3), low pI (7.3), and a very low pI (6.3-7.3) form of rbST yielded similar results. The very low pI rbST was obtained by pooling the last part of rbST eluting from the ion-exchange column. These data show the presence of monoacetylated rbST (42 Da above normal) in low pI rbST and both mono- and diacetylated species in the very low pI species. These ESMS data confirm the amino acid data and demonstrate that diacetylations can also occur in rbST.

Discussion

This work describes the identification of ϵ -N-acetyllysine in rpST and rbST, eukaryotic proteins expressed in a prokaryotic system. Initial work from our laboratory has also demonstrated the presence of this modified amino acid in 2 other recombinant eukaryotic proteins expressed in *E. coli*, bovine placental lactogen and human tissue factor pathway inhibitor (TFPI). ESMS and amino acid analyses have been used to demonstrate the presence of ϵ -N-acetyllysine in these 2 recombinant proteins. Approximately 40% of the recombinant bovine placental lactogen molecules contain ϵ -N-acetyllysine as analyzed by ESMS. Analyses of TFPI for ϵ -N-acetyllysine has been reported in a recent presentation (Leimgruber et al., 1993), which described separation of acetylated TFPI species by IPG. These results demonstrate that this modification is present in several diverse recombinant eukaryotic proteins expressed in *E. coli*. The internal location of ϵ -N-acetyllysine in proteins may explain why its identification has not been commonplace in recombinant proteins expressed in *E. coli*.



A) rpST residues labeled... residues... chromatogram... 3), which... for lysine... in the upper...

which show

used to... T and rp... rpST yield... whereas... 42 Da high... low pI... similar... the last... these data... ve normal... in the... amino acid... occur in rbST

lysine in rp... karyotic... onstrated... ombinant... ental lacto... l). ESMS... e the prese... ins. Approx... lactogen... MS. Analy... n a recent... ed separa... s demonst... e recombi... ernal loc... its ident... t protein

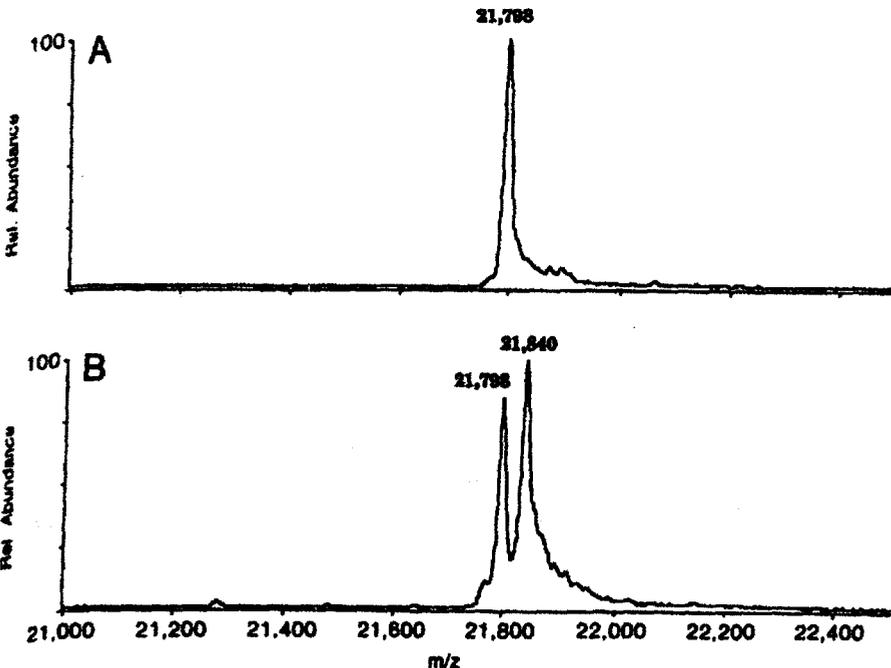


Fig. 7. Deconvoluted electrospray mass spectrometry (ESMS) spectra of (A) normal pI rpST and (B) low pI rpST shown in Figure 4. The calculated molecular weight of rpST is 21,798 amu.

RP-HPLC separation of rpST containing ϵ -N-acetyllysine¹⁴⁴ yielded the first evidence that this amino acid was present in rpST. This was the only acetylated species that was separable by RP-HPLC, indicating that this region of rpST must be involved in binding to this resin. Ion-exchange chromatography and IPG purification were used to demonstrate that this amino acid is also present at residues 112 and 171 in rpST. Harbour et al. (1992) previously demonstrated that ϵ -N-acetyllysine was present at residues 157, 167, 171, and 180 in their preparation of rbST. They

isolated a low pI fraction of rbST using chromatofocusing and then performed trypsin digestion on the entire low pI fraction without any further purification. Analyses of modified peptides from this tryptic digest yielded several peptides that were acetylated. The acetylation sites found in rbST in our work yielded this unusual amino acid at residues 144, 157, and 167. The differences in sites of acetylation in rbST between this work and previous data (Harbour et al., 1992) are probably due to the different isolation and analytical methods, different expression

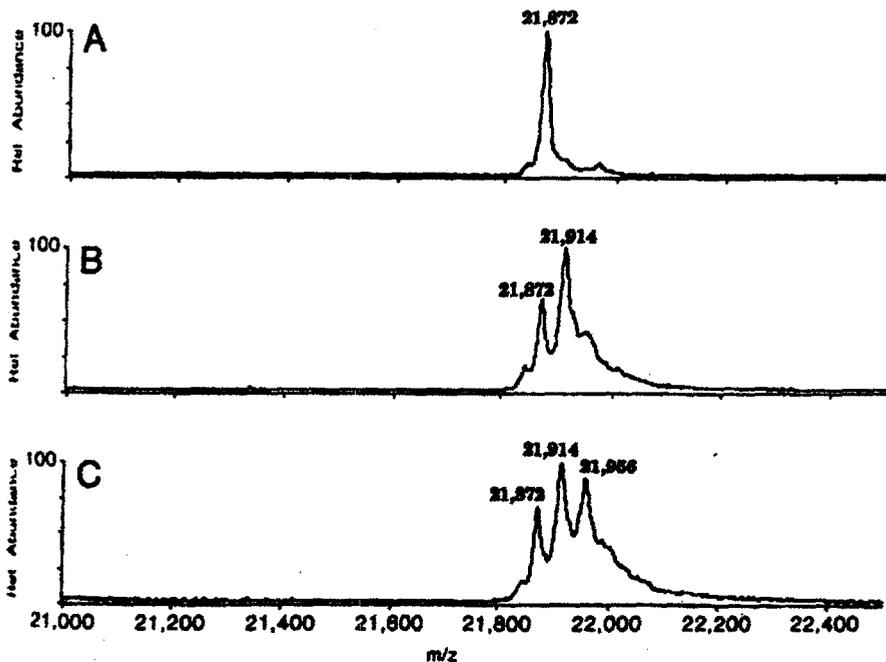


Fig. 8. Deconvoluted ESMS spectra of (A) normal pI rbST, (B) low pI rbST, and (C) a very low pI rbST. The calculated molecular weight of rbST is 21,872 amu.

levels, and differences in fermentation conditions. The effect of fermentation conditions on formation of ϵ -*N*-acetyllysine in recombinant *E. coli* proteins is currently being investigated in our laboratory and may help to explain the extent of this modification.

Amino acid sequencing, ESMS, and complete enzymatic digestion were utilized to illustrate the presence of ϵ -*N*-acetyllysine in rpST and rbST. The ESMS data (Figs. 7, 8) demonstrated that approximately half of the low pI protein is monoacetylated. For rbST, diacetylated species were also observed for the lowest pI protein analyzed. Enzymatic digestions yielded comparable data to that from ESMS with values of 0.42 and 0.54 mol of ϵ -*N*-acetyllysine per mol of rpST and rbST, respectively.

IPG has not been extensively utilized as a preparative technique for isolating modified proteins even though this technique has tremendous resolving power and can resolve proteins that differ by as little as 0.001 pI unit (Righetti & Gianazza, 1987; Gorg et al., 1988). IPG has resolved neutral hemoglobin variants, which differ by a Phe to Val substitution (Castagnola et al., 1988). Shallow pH gradients using this technique have enabled the separation of several monoacetylated species of rpST and rbST. Other techniques were unsuccessful at separating these species. Because these different monoacetylated forms have slightly dissimilar pIs (Fig. 5, lanes 3 and 4), then the microenvironment around these lysines must be sufficiently distinct to cause a difference in the pI of the proteins. Examination of the 3-dimensional structure of rpST (Abdel-Meguid et al., 1987) has shown that the modified lysines that have thus far been identified do not exist in 1 region on the molecule but are dispersed over the outside of the protein.

The manner in which rpST and rbST are acetylated has not been elucidated but is being actively investigated in our laboratory. The mechanism for formation of ϵ -*N*-acetyllysine in eukaryotic systems involves a posttranslational mechanism in which the acetyltransferase uses acetyl-CoA as the source of the acetyl group (Allfrey et al., 1984). Our attempts to demonstrate an ϵ -*N*-acetyltransferase activity in *E. coli* have been performed using a crude lysate of *E. coli* with [C^{14}]acetyl-CoA and non-acetylated rpST as a substrate. Thus far no significant incorporation of the acetyl group into rpST has been found. Utilizing the conditions described in the Materials and methods section, less than 1% incorporation of the C^{14} -acetyl group into rpST was found after 1–8 h of incubation at 37 °C. These experiments were performed both in the presence and absence of an *E. coli* lysate. The incubations were performed in the absence of the *E. coli* lysate in order to determine if the acetylation might occur by a nonenzymatic reaction. It is possible that acetylation of the lysines in rpST and rbST occurs by a chemical mechanism with acetyl-CoA or another metabolic intermediate providing the source of the acetyl group. It has been demonstrated that the acetyl group from aspirin can readily be transferred to lens protein to form ϵ -*N*-acetyllysine (Rao et al., 1985). This demonstrates the possibility that there may be a chemical intermediate at elevated concentrations (e.g., acetyl CoA) in the cell, which could chemically acetylate rpST and rbST. It has previously been shown that the metabolism of *E. coli* is altered during the expression of rbST and rpST because the unusual amino acid, nor-leucine, was synthesized and incorporated into these proteins during their expression (Bogosian et al., 1989; Violand et al., 1989a). Examination of the effect of different fermentation con-

ditions on the level of acetyl-CoA and ϵ -*N*-acetyllysine in *E. coli* is being used to determine if the metabolic state of the cell affects the level of these components.

This acetylation may be a type of signaling mechanism in *E. coli* either to direct the degradation of foreign proteins or as a signal to place proteins into inclusion bodies. It is well known that some covalent modification reactions signal a protein for degradation (Holzer & Heinrich, 1980; Levine et al., 1981; Stadtman, 1990). If formation of ϵ -*N*-acetyllysine is a signal for directing proteins into inclusion bodies, then further examination of proteins that can be expressed both in a soluble form and in inclusion bodies in *E. coli* can be used to address this important question. The mechanism by which proteins are directed into inclusion bodies has been extensively investigated, but no central theory has arisen (Kane & Hartley, 1988; Schein, 1989) although 1 publication concludes that most inclusion bodies are formed from protein-folding intermediates and not from the native or fully unfolded protein (Mitraki & King, 1989).

Four *E. coli*-derived recombinant proteins have been found to be partially α -*N*-acetylated (Grutter et al., 1985; Takao et al., 1987; Honda et al., 1989; Lischwe et al., 1993), which demonstrates that eukaryotic proteins can be α -*N*-acetylated when expressed in *E. coli*. Three native ribosomal proteins in *E. coli* are also α -*N*-acetylated and are modified by 3 different enzymes (Isono et al., 1978). The enzymes responsible for N-terminal acetylations are most likely not responsible for the side-chain acetylations found in this work because separate enzymes have been shown to be responsible for these reactions in eukaryotic systems.

It has been shown that ϵ -*N*-acetyllysine-tRNA can be used to incorporate ϵ -*N*-acetyllysine into proteins by chemically adding an acetyl group from acetoxy succinimide to the Lys-tRNA molecule (Johnson et al., 1976). ϵ -*N*-acetyllysine was incorporated at 82% efficiency into hemoglobin from ϵ -*N*-acetyllysine-tRNA in a rabbit reticulocyte cell-free protein-synthesizing system when compared to lysine incorporation from Lys-tRNA (Johnson et al., 1976). Thus, it would be possible for this modified amino acid to be incorporated into protein in *E. coli* if this tRNA derivative could be synthesized in *E. coli*.

The formation of ϵ -*N*-acetyllysine in recombinant proteins expressed in *E. coli* may limit the amount of protein that can be recovered if an ion-exchange step is used in the purification process because this process may separate these altered species. This report shows that ϵ -*N*-acetyllysine species present in rpST and rbST can be separated from the normal pI protein by ion-exchange chromatography on DE-52 resin. This chemical modification may also explain some of the heterogeneity that is generally observed in recombinant proteins. In conclusion, this research has demonstrated that acetylation of lysines is an important alteration that can occur during the expression of recombinant proteins expressed in *E. coli*.

Materials and methods

Proteins

rpST and rbST were refolded and oxidized from *E. coli* inclusion bodies as previously described (Bogosian et al., 1989). Monomeric rpST and rbST were purified from the refold/oxidation mixture using ion-exchange chromatography on a Phoenix HA-

16 Grade 3 anion exchange resin (Advanced Separation Technologies, Whippany, New Jersey). All steps were performed at 4 °C. The refolded protein (20 g) was deionized (<100 microsiemens) using 500 g of Dowex MR3 resin, adjusted to pH 10.8 with 2.5 N NaOH, and applied to the column (25 × 5 cm) equilibrated in 4.5 M urea, 0.05 M Tris-HCl, pH 10.8. The desired protein was eluted with a linear salt gradient consisting of 3.5 L of the equilibrating buffer and 3.5 L of a limit buffer of 4.5 M urea, 0.05 M Tris-HCl, 0.10 M NaCl, pH 10.8. Fractions were assayed for monomeric somatotropin (ST) using RP-HPLC and size-exclusion HPLC, which separate monomeric from dimeric protein (Violand et al., 1989b). The fractions containing less than 4% dimeric ST were pooled, extensively diafiltered against pH 10.5 H₂O (NaOH) using an Amicon Pellicon unit with a 10,000 molecular weight membrane, and lyophilized.

Purification of low pI forms of rpST and rbST

Samples enriched in the low pI forms of rpST and rbST were prepared by chromatography of the product from the Phoenix ion-exchange column on a Whatman DE-52 resin. The sample was dissolved at 10 mg/mL in 4.5 M urea, 0.05 M Tris-HCl, pH 10.7, and chromatographed as described previously (Wood et al., 1989). Fractions of the low pI ST were pooled based on analyses of fractions by IEF. The pooled sample was diafiltered extensively against pH 10.5 H₂O (NaOH) with an Amicon Pellicon unit with a 10,000 molecular weight membrane and lyophilized.

Reversed-phase HPLC separation of rpST containing ε-N-acetyllysine¹⁴⁴

A Perkin-Elmer Series 4 HPLC was used for this separation on a Vydac C-18 column (218TP510) at a flow rate of 5 mL/min. The protein (500 μg) was applied at 40 mM trifluoroacetic acid (TFA), 55% acetonitrile, and eluted with a linear gradient from 55 to 61% acetonitrile over 24 min at constant 40 mM TFA. Protein in the eluate was monitored at 278 nm. Data were collected and stored using a Nelson 4400 chromatography data system.

Amino acid analysis

Amino acid analyses were performed as previously described (Violand et al., 1990).

Amino acid sequencing

Automated amino acid sequencing was performed as previously described (Violand et al., 1990).

Preparation of PTH-ε-N-acetyllysine

An aqueous solution of ε-N-acetyllysine was prepared and applied to an underivatized TFA-etched, GF/C filter disc (Applied Biosystems, Inc., Foster City, California). It was then derivatized with phenylisothiocyanate (PITC) reagent in a model 470A gas-phase sequencer (Applied Biosystems, Inc.) and converted into the stable PTH form using the standard sequencing protocol. The derivative was delivered in an on-line fashion to a model 120A PTH-Analyzer (Applied Biosystems, Inc.) in order

to establish its retention time relative to the other PTH-amino acid derivatives.

Tryptic peptide mapping

Tryptic peptide mapping was performed as previously described (Violand et al., 1990).

Fast-atom bombardment mass spectrometry (FAB-MS)

Positive FAB-MS was performed using FAB-MS on a Finnigan TSQ mass spectrometer equipped with an Iontech FAB source as previously described (Violand et al., 1990).

Complete enzymatic digestion and amino acid analyses for quantitation of ε-N-acetyllysine

The method used for determining the amount of ε-N-acetyllysine in somatotropins was essentially identical to a method previously published for this determination in histones with 1 modification being that norleucine was included as an internal standard (Allfrey et al., 1984).

Carboxymethylation and RP-HPLC purification of rpST and rbST from E. coli inclusion bodies

Inclusion bodies of rpST or rbST were prepared as previously described (Bogosian et al., 1989). The inclusion bodies were solubilized at 30 mg/mL of total protein in 5% lithium dodecyl sulfate, 0.1 M Tris-HCl, 0.05 M ethylenediamine tetraacetate, pH 8.6. An aliquot of β-mercaptoethanol was added to a final concentration of 0.27 M, and the sample was incubated for 15 min at 37 °C. After reduction, iodoacetate was added so that it was 0.05 M excess over the amount of β-mercaptoethanol, and the pH was maintained at 8.6 for 15 min at room temperature with addition of 2.5 N NaOH. The reaction was stopped by addition of 0.1 M β-mercaptoethanol. The reduced, carboxymethylated rpST or rbST was then purified by RP-HPLC. The protein (1 mg) was applied to a Synchrom RP-8 column (250 × 10 mm) equilibrated in 48% acetonitrile, 20 mM TFA. The desired protein was then eluted by holding at 48% acetonitrile for 5 min, ramped using a linear gradient to 75% acetonitrile over 1 min, and held at 75% acetonitrile for 4 min. The flow rate was 6 mL/min, and the column eluate was monitored for protein at 278 nm.

Isoelectric focusing

Isoelectric focusing was performed as previously described (Violand et al., 1992).

Immobilized pH gradient electrophoresis

IPG of rpST was performed using a pH 6–10 gradient in 3 M urea according to published procedures (Righetti & Gianazza, 1987). For analytical purposes, approximately 100 μg of sample was analyzed. For preparative purposes, a sample application trough was made in the gel by applying 2 layers of cellophane tape approximately 2 cm from the bottom of the U-frame plate.

A sharp knife was used to remove strips from the top and bottom edges of the tape such that the tape was 5 cm wide and 1.8 cm from the edge of the gasket and was 5 mm wide. The gel was poured in the normal fashion, and the tape made a trough in the gel where volumes of up to 500 μ L (10–20 mg of protein) of sample were applied. A single gel was run at 3,000 V (max), 5.0 mA (max), and 5.0 W (max) at 10 °C. The focusing time was approximately 15 h. Protein bands were cut out of the gel by visual inspection. The protein was extracted from the gel using 1–2 mL of 2% lithium dodecyl sulfate, 0.2 M Tris-HCl, 0.01 M EDTA, pH 8.6. This mixture was stirred overnight at 4 °C, and the protein was isolated by centrifuging the sample and performing RP-HPLC as described above for purification of reduced-carboxymethylated rpST from inclusion bodies.

Assay for acetylation of pST using C^{14} -acetyl-CoA

A crude lysate of *E. coli* cells was made by using 3 passes through an APV Gaulin homogenizer. Nonacetylated rpST (as analyzed by ESMS) was purified using the same DE-52 column procedure utilized for purifying the low pI forms of rpST. This pST was dissolved at 3 mg/mL in 0.1 M Tris-HCl, 0.005 M EDTA, 0.01 M KCl, 20% glycerol, 0.005 M β -mercaptoethanol, pH 8.0. This pST (0.10 mL) was added to 0.10 mL of the crude *E. coli* extract, and the reaction was initiated by adding 0.10 mL of C^{14} -acetyl CoA (0.60 mM) purchased from NEN. At the desired time points, 120 μ L was removed and placed onto a Synchrom C-8 column equilibrated in 43% acetonitrile, 20 mM TFA. The rpST was eluted using a linear acetonitrile gradient from 43 to 53% over 10 min at constant 20 mM TFA. The flow rate was 2 mL/min and the column eluate was monitored at 280 nm. The collected pST was then counted using a liquid scintillation counter. For experiments without the crude *E. coli* lysate, 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.4, was substituted for the crude lysate.

ESMS

These analyses were performed as previously described (Violand et al., 1992).

Acknowledgments

We acknowledge the technical assistance of Patti L. Morris and the preparation of bovine placental lactogen by Daniel F. Curran. We also acknowledge the support of Gerald L. Bachman and Clifton A. Baile during the course of this work.

References

- Abdel-Meguid SS, Shieh HS, Smith WW, Dayringer HE, Violand BN, Bentle LA. 1987. Three-dimensional structure of a genetically engineered variant of porcine growth hormone. *Proc Natl Acad Sci USA* 84:6434–6437.
- Allfrey VG, Faulkner R, Mirsky AE. 1964. Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proc Natl Acad Sci USA* 51:786–794.
- Allfrey VG, Ki Paola EA, Sterner R. 1984. Protein side-chain acetylations. *Methods Enzymol* 107:224–240.
- Bogosian G, Violand BN, Dorward-King EJ, Workman WE, Jung PE, Kane JF. 1989. Biosynthesis and incorporation into protein of norleucine by *Escherichia coli*. *J Biol Chem* 264:531–539.
- Castagnola M, Dobosz M, Landolfi R, Pascali VL, Angelis F, Vettore L, Perona G. 1988. Determination of neutral hemoglobin variants by immobilized pH gradients. *Biol Chem Hoppe-Seyler* 369:241–246.
- Csordas A. 1990. On the biological role of histone acetylation. *Biochem J* 265:23–38.
- Delange RJ, Fanbrough DM, Smith EL, Bonner J. 1969. The complete amino acid sequence of calf thymus histone IV, presence of ϵ -N-acetyllysine. *J Biol Chem* 244:319–334.
- Edde B, Rossier J, Le Caer JP, Berwald-Netter Y, Koulakoff A, Gros F, Denoulet P. 1991. A combination of posttranslational modifications is responsible for the production of neuronal α -tubulin heterogeneity. *J Cell Biochem* 46:134–142.
- Gershey EL, Vidali G, Allfrey VG. 1968. Chemical studies of histone acetylation. *J Biol Chem* 243:5018–5022.
- Gorg A, Fawcett JS, Chramback A. 1988. The current state of electrofocusing in immobilized pH gradients. *Adv Electrophoresis* 2:1–47.
- Grueter MG, Marki W, Walliser HP. 1985. Crystals of the complex between recombinant N-acetylglucosyl C and subtilisin. *J Biol Chem* 260:11436–11437.
- Harbour GC, Garlick RL, Lyse SB, Crow FW, Robins, RH, Hoogerheide JG. 1992. N- ϵ -acetylation can occur at lysine residues 157, 167, 171 and 180 of recombinant bovine somatotropin. *Techniques Protein Chem* 3:487–495.
- Hase T, Wakabayashi S, Matsubara H, Kerscher L, Oesterheld D, Rao KK, Hall DO. 1978. Completed amino acid sequence of *Halobacterium halobium* ferredoxin containing an ϵ -N-acetyllysine. *J Biochem* 83:1657–1659.
- Holzer H, Heinrich PC. 1980. Control of proteolysis. *Annu Rev Biochem* 49:63–91.
- Honda S, Asano T, Kajio T, Nishimura O. 1989. *E. coli* derived human interferon- γ with Cys-Tyr-Cys at the N-terminus is partially N- α -acetylated. *Arch Biochem Biophys* 269:612–622.
- Isono S, Isono K, Hirota Y. 1978. Mutations affecting the structural genes and the genes coding for modifying enzymes in ribosomal proteins of *E. coli*. *Mol Gen Genet* 165:15–20.
- Johnson AE, Woodward WR, Herbert E, Menninger JR. 1976. N⁶-acetyllysine transfer ribonucleic acid: A biologically active analogue of aminoacyl transfer ribonucleic acids. *Biochemistry* 15:569–575.
- Kane JF, Hartley DL. 1988. Formation of recombinant inclusions in *E. coli*. *Trends Biotechnol* 6:95–101.
- Leimgruber RL, Junger KD, Gustafson ME, Violand BN. 1993. Characterization of human tissue factor pathway inhibitor by immobilized pH gradient electrophoresis. *Proc Electrophoresis Soc. Abstr* 112.
- Levine RL, Oliver CN, Fulks RM, Stadtman ER. 1981. Turnover of bacterial glutamine synthetase: Oxidative inactivation precedes proteolysis. *Proc Natl Acad Sci USA* 78:2120–2124.
- L'Hernault SW, Rosenbaum T. 1985. Reversal of the posttranslational modification on *Chlamydomonas* flagellar α -tubulin occurs during flagellar resorption. *Biochemistry* 24:473–478.
- Lischwe MA, Newton RC, Huang JJ, Yates RA, Breth LA, Larsen BS. 1993. *Escherichia coli* derived murine interleukin-13 with N-terminus partially N- α -acetylated. *Protein Express Purif* 4:499–502.
- Mitraki A, King J. 1989. Protein folding intermediates and inclusion body formation. *Biotechnology* 7:690–697.
- Nelson DA. 1982. Histone acetylation in bakers yeast. *J Biol Chem* 257:1565–1568.
- Nohara J, Takahashi T, Ogata K. 1966. Acetylation of histones by pigeon liver enzymes. *Biochim Biophys Acta* 127:282–290.
- Piperno G, LeDizet M, Chang X. 1987. Microtubules containing acetylated α -tubulin in mammalian cells in culture. *J Cell Biol* 104:289–302.
- Rao GN, Lardis MP, Cotlier E. 1985. Acetylation of lens crystallins: A possible mechanism by which aspirin could prevent cataract formation. *Biochem Biophys Res Commun* 128:1125–1132.
- Righetti PR, Gianazza E. 1987. Isoelectric focusing in immobilized pH gradients: Theory and newer methodology. *Methods Biochem Anal* 32:215–278.
- Ruiz-Carrillo A, Wanh LJ, Allfrey VG. 1976. Selective synthesis and modification of nuclear proteins during maturation of avian erythroid cells. *Arch Biochem Biophys* 174:273–290.
- Schein CH. 1989. Production of soluble recombinant proteins in bacteria. *Biotechnology* 7:1141–1149.
- Stadtman ER. 1990. Covalent modification reactions are marking steps in protein turnover. *Biochemistry* 29:6323–6331.
- Sterner R, Vidali G, Allfrey VG. 1979. Studies of acetylation and deacetylation in high mobility group proteins. *J Biol Chem* 254:11577–11583.
- Sterner R, Vidali G, Allfrey VG. 1981. Studies of acetylation and deacetylation in high mobility group proteins. *J Biol Chem* 256:8892–8895.
- Takao T, Kobayashi M, Nishimura O, Shimomishi Y. 1987. Chemical char-

Vectors
 nts by
 246.
 Biochem
 ete
 .tyllysine
 A. Gros
 fications
 eity. *J Cell*
 istone
 of elect
 s 2:1-47.
 lex betwe
 260:1143
 oogerhel
 67, 171
 otein Chem
 D. Rao K
 ictarium
 m 83:1657
 ev Biochem
 ived human
 y N-acetyl
 ctural ge
 l proteins
 5. N⁶-acetyl
 of aminoac
 ns in *E. coli*
 3. Character
 ized pH ge
 2.
 ver of bac
 s proteoly
 lational me
 uring flagel
 arsen BS. 19
 minus parti
 inclusion bod
J Biol Chem
 es by pigso
 ing acetyl
 289-302.
 allins: A pos
 t formation
 ized pH ge
 em Anal 32:
 esis and man
 erythroid cell
 ns in bacter
 arking steps
 n and deac
 :11577-115
 n and deac
 :8892-889
 Chemical ch

acterization of recombinant human leukocyte interferon A using fast atom bombardment mass spectrometry. *J Biol Chem* 262:3541-3547.
 Violand BN, Schlittler MR, Kolodziej EW, Toren PC, Cabonce MA, Siegel NR, Duffin KL, Zobel JF, Smith CE, Tou JS. 1992. Isolation and characterization of porcine somatotropin containing a succinimide residue in place of aspartate¹²⁹. *Protein Sci* 1:1634-1641.
 Violand BN, Schlittler MR, Toren PC, Siegel NR. 1990. Formation of iso-aspartate 99 in bovine and porcine somatotropins. *J Protein Chem* 9:109-117.
 Violand BN, Siegel NR, Bogosian G, Workman WE, Kane JF. 1989a. De-

tection of norleucine incorporation into recombinant proteins synthesized in *E. coli*. *Techniques Protein Chem*. pp 315-326.
 Violand BN, Takano M, Curran DF, Bentle LA. 1989b. A novel concatenated dimer of recombinant bovine somatotropin. *J Protein Chem* 8: 619-628.
 Wood DC, Salsgiver WJ, Kasser TR, Lange GW, Rowold E, Violand BN, Johnson A, Leimgruber RM, Parr GR, Siegel NR, Kimack NM, Smith CE, Zobel JF, Ganguli SM, Garbow JR, Bild G, Krivi GG. 1989. Purification and characterization of pituitary bovine somatotropin. *J Biol Chem* 264:14741-14747.

Forthcoming Papers

Ligation alters the pathway of urea-induced denaturation of the catalytic trimer of *Escherichia coli* aspartate transcarbamylase

S. Bromberg, V.J. LiCata, D. Mallikarachchi, and N.M. Allewell

Primary structure and functional characterization of rat C5a: An anaphylatoxin with unusually high potency

L. Cui, D.F. Carney, and T.E. Hugli

Laue and monochromatic diffraction studies on catalysis in phosphorylase *b* crystals

E.M.H. Duke, S. Wakatsuki, A. Hadfield, and L.N. Johnson

Collectins—soluble proteins containing collagenous regions and lectin domains—and their roles in innate immunity

H.-J. Hoppe and K.B.M. Reid

Protein identification in DNA databases by peptide mass fingerprinting

P. James, M. Quadroni, E. Carafoli, and G. Gonnet

Extending the B7 (CD80) gene family

P.S. Linsley, R. Peach, P. Gladstone, and J. Bajorath

A survey of furin substrate specificity using substrate phage display

D.J. Matthews, L.J. Goodman, C.M. Gorman, and J.A. Wells

Derivation of rules for comparative protein modeling from a database of protein structure alignments

A. Sali and J.P. Overington

Deuterium exchange of alpha-helices and beta-sheets as monitored by electrospray ionization mass spectrometry

D.S. Wagner, L.G. Melton, Y. Yan, B.W. Erickson, and R.J. Anderegg

Properties of a recombinant human hemoglobin with aspartic acid 99(β), an important intersubunit contact site, substituted by lysine

H. Yanase, S. Cahill, J.J. Martin de Llano, L.R. Manning, K. Schneider, B.T. Chait, K.D. Vandegriff, R.M. Winslow, and J.M. Manning

Two-step selective formation of three disulfide bridges in the synthesis of the C-terminal epidermal growth factor-like domain in human blood coagulation factor IX

Y. Yang, W.V. Sweeney, K. Schneider, B.T. Chait, and J.P. Tam

bert Cohen
0 Oradell Ave.
adell, NJ 07649

CERTIFIED

Z 338 911 901

MAIL



Dockets Management Branch
Food and Drug Administration
Department of Health and Human Services
Room 1-23
12420 Parklawn Dr.
Rockville, MD 20857